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IN VITRO ASSESSMENT OF THE TOXICITY OF COCAINE
AND ITS METABOLITES IN THE HUMAN UMBILICAL ARTERY

A Dissertation

Presented to the Faculty of
the Department of Pharmacology
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Science

by

Tessa Lea Long

August 1998

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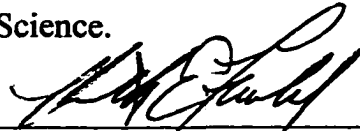
This is to certify that the Graduate Committee of

TESSA LEA LONG

Met on the

21st day of July, 1998.

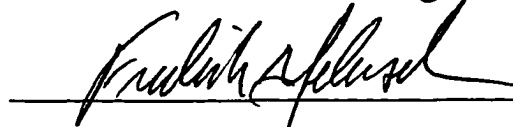
The committee read and examined her dissertation, supervised her defense of it in an oral examination, and decided to recommend that her study be submitted to the Graduate Council, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science.



Chairman, Graduate Committee



Co-chairman



Signed on behalf of the
Graduate Council



Dean of Graduate Studies

ABSTRACT

IN VITRO ASSESSMENT OF THE TOXICITY OF COCAINE

AND ITS METABOLITES IN THE HUMAN UMBILICAL ARTERY

by

Tessa L. Long

An in vitro model was used to assess the effect of cocaine and its metabolites on the umbilical artery. Objectives were to pharmacologically confirm the presence of adrenergic innervation using tyramine, evaluate the ability of cocaine, benzoylecgonine, norcocaine and cocaethylene to potentiate vasoconstriction by serotonin and norepinephrine, examine the ability of ketanserin to block the enhanced vasoconstriction produced by cocaine, and determine displacement of ³H-ketanserin by serotonin, norepinephrine, tyramine and mianserin.

The vasoconstrictive effect of tyramine (100 μ M) was enhanced in the presence of cocaine by 257%. Vasoconstrictive effects of serotonin and norepinephrine were significantly enhanced by cocaine by 28%, and 64% respectively; producing significant increases in the cumulative response. Norcocaine significantly augmented the maximum response to norepinephrine by 54%. Benzoylecgonine significantly decreased the maximum response to serotonin by 36% as well as the cumulative response.

Ketanserin (0.03 μ M) completely attenuated the vasoconstrictive potentiation of serotonin and norepinephrine by cocaine; shifting the EC₅₀ for serotonin to the right 10-fold in the presence of ketanserin and cocaine. Ketanserin shifted the EC₁₅ for norepinephrine with cocaine to the right 205-fold. Maximum response to norepinephrine with cocaine was depressed 54% by ketanserin.

Serotonin, tyramine, and mianserin were able to displace ³H-ketanserin (3 nM) from the membrane fraction of the human umbilical artery. This indicates serotonin₂ receptor involvement in vasoconstrictive responses to serotonin and tyramine. Norepinephrine did not displace ³H-ketanserin in the membrane fraction of the umbilical artery.

These data suggest that enhanced vasoconstriction of norepinephrine and serotonin by cocaine and potentiation of the maximum response to norepinephrine by norcocaine in the human umbilical artery may be important components of perinatal cocaine toxicity. Ketanserin was able to suppress the umbilical artery constriction produced by cocaine, demonstrating its antidotal potential. The potentiation of the tyramine response by cocaine and the displacement of ^3H -ketanserin by tyramine indicate that tyramine may be producing its vasoconstrictive effect through serotonin₂ receptors in the human umbilical artery.

INSTITUTIONAL REVIEW BOARD APPROVAL

This is to certify that the following study has been filed and approved by the Institutional Review Board of East Tennessee State University.

Project Title: Effects of Cocaine, Norcocaine, and Cocaethylene on the Isolated Umbilical Artery and Vein, and the Influence of Progesterone.

Principle Investigator: Kenneth E. Ferslew, Ph.D.

Department: Pharmacology, Section of Toxicology

Date Submitted: August 15, 1996

Institutional Review Board Chair: David N. Walters, M.D.

DEDICATION

In loving memory of Agnes E. Poe (1905-1993).

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Many thanks to the faculty and staff of the Department of Pharmacology for all the advice, support, and knowledge so graciously bestowed upon me.

Thanks to the members of my committee for their patience and efforts on my behalf.

Successful completion of this project would not have been possible without the generous assistance of Dr. Peter Rice, Ph.D. I am particularly grateful for the use of his laboratory as well as his guidance.

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CHAPTER 1

INTRODUCTION

Survey of Deleterious Perinatal Cocaine Effects

Human epidemiological data has consistently implicated cocaine use during pregnancy with various perinatal and postnatal complications. Infants born to cocaine abusing mothers suffer many complications including genito-urinary anomalies (Chasnoff et al. 1988), cerebral infarct (Chasnoff et al. 1986), and ischemic infarct of the bowel (Telsey et al. 1988) as well as various other anomalies. A study conducted by MacGregor et al. (1987) surveyed the outcome of 70 newborns, those infants exposed to cocaine in utero presented with significantly lower birth weights as well as increased incidence of prematurity, preterm delivery, and small for gestational age newborns. Perinatal cocaine abuse often compromises the pregnancy itself with increased incidence of placental abruption (Acker et al. 1983; Chasnoff et al. 1985; Oro and Dixon 1987), stillbirths (Bingol et al. 1987), spontaneous abortion, and preterm labor (Chasnoff et al. 1985; Chasnoff et al. 1987; MacGregor et al. 1987; Collins et al. 1989). These effects seem to be predominantly related to the vasoconstrictive and hypertensive actions of cocaine (Plessinger and Woods 1991).

Animal models have provided useful information concerning cocaine mechanisms which contribute to deleterious perinatal and postnatal effects. Placental vasoconstriction as a consequence of cocaine administration has been observed in a mouse model by Mahalik et al. (1984) and by Malek et al. (1995) in isolated term human placenta. Several studies have confirmed the ability of cocaine to reduce uterine blood flow. Among the first to conduct such studies was Mahalik et al. (1984), who observed this decrease in blood flow in response to norepinephrine mediated vasoconstriction in the pregnant ewe. They demonstrated this concept experimentally by incorporating tropacocaine, an agent similar to cocaine which does not inhibit the reuptake of norepinephrine into the nerve terminal. In cocaine-exposed animals, there was a decrease in the amount of radio labeled sodium transferred to the embryos. However, in the tropacocaine-exposed animals the transfer of the isotope was not reduced significantly. In response to intravenous cocaine administered to the uterine vasculature of the pregnant ewe (Woods et al. 1987; Moore et al. 1986), and to the pregnant nonhuman primate (Morgan et al. 1991), a dose-dependent reduction of uterine blood flow was observed. This results in increased blood pressure and restricts the delivery of essential nutrients and oxygen to the fetus.

Moreover, it has been established that cocaine readily crosses the placenta, producing increases in fetal heart rate and blood pressure in the pregnant ewe

(Woods et al. 1987). Numerous studies conducted in animal models indicate that cocaine and its metabolites are rapidly transferred across the placenta to the fetal compartment (Moore et al. 1986; Woods et al. 1989). Schenker et al. (1993) observed that cocaine and its metabolites are transferred across the isolated human placenta almost as rapidly as antipyrine, a substance which is used as a marker for placenta flow. These observations suggest that cocaine may exert its detrimental effects not only by producing fetal hypoxemia through reduction of blood flow to the uterine and placental vasculature, but also by crossing the placenta to have direct effects on the fetus.

Several of the perinatal and postnatal cocaine effects observed in animal models have been linked to similar effects observed in human epidemiological data. These effects are produced by vasoconstriction and include placental abruption, placental constriction, reduced uterine blood flow and decreased viability of the fetus. Animal model correlations provide some insight concerning the perinatal toxic potential of cocaine, however, the full impact of continuous cocaine abuse throughout the pregnancy remains to be elucidated.

Survey of Cocaine Effect on the Human and Ovine Umbilical Artery

The umbilical vasculature is an important component of the maternal-fetal unit which has not been carefully assessed in terms of its contribution to the

vasoconstrictive effects associated with cocaine abuse during pregnancy. Dyer (1970) produced a dose-dependent potentiation of serotonin vasoconstriction by cocaine in isolated strip preparations of ovine umbilical arteries, but saw no such potentiation of norepinephrine responses. In the isolated guinea pig umbilical artery, Nair and Dyer (1974) found that cocaine potentiated the contractions produced by serotonin and norepinephrine, shifting the dose-response curves to the left three-fold. Hoskins et al. (1991) performed umbilical artery Doppler flow velocimetry studies on 314 women who had a history of cocaine abuse, and placental abruption or preterm labor and compared these women with controls presenting with placental abruption or preterm labor but did not use cocaine. They reported cocaine abuse associated with placental abruption, preterm birth, and low birth weight was correlated with increased systolic/diastolic ratios in human umbilical artery blood flow in some women. Recently, Zhang and Hu (1998) demonstrated that cocaine potentiates isolated ovine umbilical artery contractions produced by serotonin in the endothelium-intact preparation. These studies provide evidence that cocaine may directly potentiate constriction of the umbilical artery.

Bodelsson and Stjernquist (1995) did not observe a potentiation of norepinephrine by cocaine in the isolated human umbilical artery. This suggests that serotonin may be more important to umbilical artery vasoconstriction as

a possible contributing factor in the morbidity and mortality associated with cocaine exposure to the developing fetus.

Pharmacological Mechanism of Cocaine

The adverse perinatal and neonatal outcomes associated with cocaine abuse during pregnancy have generally been attributed to the vasoconstrictive effects of cocaine and its metabolites. This vascular effect is thought to be related to the ability of cocaine and its metabolites to block the reuptake of norepinephrine at the presynaptic nerve terminal, inhibiting the primary means of inactivation for this neurotransmitter (Furchgott et al. 1963).

Several very elegant studies have contributed to what is now known about the pharmacological actions of cocaine. A study conducted by Lockett and Eakins (1960) revealed that cocaine was able to abolish the pressor response to tyramine in cats. Since tyramine is an indirect acting sympathomimetic which produces its effects by displacing norepinephrine from presynaptic nerve terminals, the results of this study indicate that cocaine exerts its effect by altering the transport of catecholamines into the nerve terminal. This mechanism was first described by Kirpekar et al. (1962), who proposed that cocaine blocks a specific "transfer site". Furchgott et al. (1963), Trendelenburg (1965), Johnson and Kahn (1966), and Barnett et al. (1968) have provided definitive evidence that cocaine blocks the

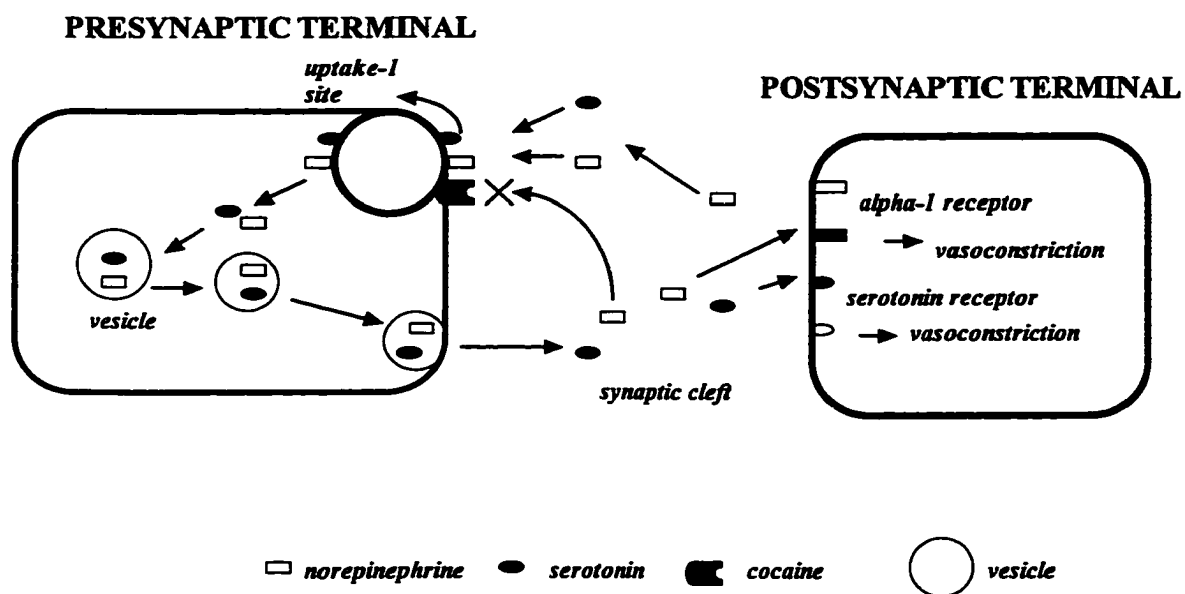


Figure 1. Diagrammatic representation of the pharmacological mechanism of cocaine.

synaptic reuptake of catecholamines, increasing norepinephrine levels and post-synaptic stimulation of adrenergic receptors (Bayorh et al. 1983) (Figure 1).

Systemic pressor effects of cocaine administration are produced by increased plasma levels of norepinephrine which increase systemic vascular resistance. Thus, many of the toxic effects associated with perinatal cocaine abuse may be due to its hemodynamic alterations. If the response of the umbilical vasculature is similar to that of other tissues, vasoconstriction of this tissue could contribute to the reduced perfusion of the developing fetus resulting in many of the previously described perinatal complications.

Survey of the Vascular Effects of Cocaine Metabolites

Cocaine is extensively metabolized in the plasma and liver, its metabolites are cleared from the plasma more slowly than cocaine itself (Jatlow 1987). Several of the resulting metabolites exhibit pharmacological activity similar to that of cocaine. Benzoylecgonine is the major metabolite (80%) of cocaine formed after hydrolysis by nonspecific plasma and liver esterases. Benzoylecgonine is reported to possess pharmacological activity to produce vasoconstriction of cat cerebral arteries (Madden and Powers 1990). Cocaethylene (Jatlow et al. 1991) is a toxic metabolite of cocaine formed by the liver when cocaine and ethanol are used concomitantly by benzoylecgonine carboxylesterase (Boyer and Petersen 1991), the amount of cocaethylene formation is dependent on plasma concentration of ethanol. The presence of ethanol shifts metabolism of cocaine from benzoylecgonine to cocaethylene and increases the formation of norcocaine (Farre et al. 1993). Cocaethylene is purported to be more cardiotoxic than cocaine (Randall 1992) and exhibits a lower LD50 in mice (Hearn et al. 1991). Norcocaine is formed by N-demethylase in human liver and accounts for 20% of cocaine metabolism (Stuart et al. 1979). Norcocaine is more efficacious than cocaine itself at inhibiting synaptic reuptake of norepinephrine (Hawks et al. 1975). In one experiment involving porcine fetal cerebral arteries, cocaethylene was able to produce vasoconstriction in these vessels more efficaciously than cocaine. In the

same study, norcocaine was shown to constrict these vessels as efficaciously as cocaine itself (Kurth et al. 1993). Similar effects were described in fetal lambs, Covert et al. (1994) reported increased cerebral vascular resistance in response to cocaethylene and benzoylecgonine administration.

During pregnancy, the toxicity of cocaine and its metabolites is greater due to decreased activity of cytochrome P₄₅₀ enzymes and increased plasma levels of progesterone (Neale and Parks 1973). Progesterone increases enzymatic demethylations thereby increasing plasma concentrations of longer-lived toxic cocaine metabolites and by increasing vascular reactivity to alpha-adrenergic receptor agonists. Pregnancy itself enhances cocaine sensitivity by increasing the formation of norcocaine and cocaethylene while decreasing norcocaine metabolism. In one study, the cardiovascular toxicity to cocaine was shown to be increased in the presence of progesterone in nonpregnant ewes (Plessinger and Woods et al. 1990).

Question of Innervation in the Human Umbilical Cord

The subject of innervation in the umbilical vasculature has been a topic of controversy for several decades. Even as recently as 1990 the conflict continued; it was demonstrated that adrenergic and cholinergic nerve fibers are present in umbilical arteries at the fetal end of the human umbilical cord using histochemical

techniques (Kawano and Mori 1990). In the same year another study reported no evidence of innervation using immunohistological and histochemical techniques (Fox and Khong 1990).

Functional in vitro studies have provided some information that supports the existence innervation of human umbilical arteries. Gulati and Kelkar (1970) found that cocaine potentiates the responses of norepinephrine and serotonin in a human whole cord perfusion study. They also found that tyramine produced vasoconstriction of the artery and this response was potentiated by cocaine. These findings suggest that the human umbilical cord is innervated and that cocaine and its metabolites potentiate responses to serotonin and norepinephrine thereby contributing to toxic vasoconstrictive situations produced with perinatal cocaine abuse.

Question of Receptor Types Present in Human Umbilical Arteries

Norepinephrine produces contractions of less than half the KCl maximum in isolated umbilical arteries even at high concentrations (Bodelsson and Stjernquist 1995). This is a very unusual response for arterial tissue which is normally characterized as primarily alpha-adrenoceptor mediated. Bodelsson and Stjernquist (1995) were able to produce contractions of isolated human umbilical artery rings with oxymetazoline, an α_2 agonist, however the maximum

response was five times higher than that of norepinephrine itself suggesting that oxymetazoline produced its effects through another receptor. The authors concluded that oxymetazoline was probably acting through serotonin receptors since methysergide, a nonspecific serotonin antagonist, produced a parallel shift in the concentration-effect curve for oxymetazoline. In the same study, the authors noted that prazosin (α_1 antagonist) produced concentration-dependent reductions in the concentration-effect curves to norepinephrine, but not a parallel shift of the response. The authors concluded that norepinephrine produced its effects through both α_1 and α_2 receptors. Adding to the confusion, Tuncer et al. (1985) were not able to produce contractions of this tissue at all with clonidine, an α_2 agonist. These observations suggest that norepinephrine may be producing its effects through another receptor type, possibly serotonin receptors.

Serotonin is a very potent vasoconstrictor of human umbilical arteries (Gokhale et al. 1966; Altura et al. 1972; Tuncer et al. 1985; McGrath et al. 1988; Yoshikawa and Chiba 1991) and this response is antagonized by the serotonin₂ receptor antagonists ketanserin and mianserin (Tuncer et al. 1985). The pA_2 values (slope of the regression of K_D values obtained for an agonist over several concentrations of antagonist) reported in Tuncer's study in the human umbilical artery for ketanserin and mianserin were 7.89 and 8.06. These values were

significantly lower than those reported for other tissues; including rat jugular vein (Cohen et al. 1983) and rat uterus (Ichida et al. 1983), where observed mianserin pA_2 values were 9.30 and 9.70, and in the same tissues as well as calf coronary arteries (Kaumann 1983) and rat tail arteries (Hicks and Langer 1983) ketanserin pA_2 values ranged from 9.10-9.80. Based on these numbers Tuncer et al. (1985) concluded that serotonin induced contractions in the isolated human umbilical artery were mediated by neither serotonin₁ nor serotonin₂ receptors.

These contributions to the literature concerning the receptor types responsible for norepinephrine and serotonin mediated contractions of isolated human umbilical arteries leave many unanswered questions about the serotonin and alpha₁ adrenoceptor population present in this tissue. This information is vital to the understanding of how cocaine might be exerting its effects and what compounds might be useful in antagonizing cocaine potentiation of norepinephrine and serotonin vasoconstriction.

Role of Ketanserin in the Treatment of Hypertension

Serotonin seems to play an important role in the sequelae of preeclampsia-eclampsia. Several studies have noted the benefit of ketanserin therapy in the treatment of this perinatal complication (Ghisoni 1990; Rossouw et al. 1995; Steyn and Odendaal 1997). Rossouw et al. (1995) found ketanserin to be a safer

alternative to hydralazine therapy in late term hypertension and Steyn and Odendaal (1997) found ketanserin to be a beneficial addition to low dose aspirin therapy among women with mild to moderate midtrimester hypertension. Ketanserin has also been found to be useful in other types of hypertensive situations (Van Neuten et al.1981; DeCree et al. 1981; Symoens and Janssen 1986). Tuncer et al. (1985) did notice that ketanserin produced effects on isolated human umbilical artery strips, significantly shifting concentration-effect curves to serotonin. This information opens the possibility of a role for ketanserin in perinatal hypertensive situations produced by cocaine abuse.

Statement of the Problem

Human epidemiological data, whole animal studies, and isolated tissue studies have confirmed that cocaine produces vasoconstrictive situations which contribute to the well documented deleterious consequences of perinatal cocaine abuse. The contribution of placental and uterine vasoconstriction has been established in animal models and in isolated perfused placentas. The constriction of umbilical vessels in this situation has not been studied as carefully. Research to date indicates that cocaine may produce potentiation of serotonin and/or norepinephrine mediated vasoconstriction in the human umbilical artery, further

exacerbating the reduced perfusion of the fetus produced by the constriction of the uterine artery and the placental vasculature.

Requisite to a situation whereby cocaine produces a vasoconstrictive or hypertensive situation in the umbilical artery via its conventionally described mechanism is the presence of nerve terminals in the smooth muscle of the vasculature. There is histological (Kawano and Mori 1990) and functional (Gulati and Kelkar 1970) data to indicate that human umbilical arteries are innervated. However, this tissue is usually described as non-innervated due to previous histological studies which were unable to detect nerve endings in the human umbilical cord. Central to understanding the mechanisms by which cocaine might produce vasoconstriction in this tissue is determination of innervation.

The ability of cocaine to potentiate norepinephrine induced vasoconstriction in the human umbilical artery is disputed by Bodelsson and Stjernquist (1995) and supported by Gulati and Kelkar (1970). Gulati and Kelkar (1970) found that cocaine also potentiated the vascular response of serotonin in the whole perfused human umbilical cord. Zhang and Hu (1998) noted the same effect in isolated ovine umbilical artery rings. This effect as well as the impact of pharmacologically active circulating metabolites of cocaine requires further elucidation.

Several experiments have failed to reveal definitive information about the receptor types responsible for the vasoactive effects of norepinephrine and serotonin in the umbilical artery. Bodelsson and Stjernquist (1995) concluded that α_1 and α_2 adrenoceptors are responsible for norepinephrine induced contractions of isolated human umbilical artery rings even though they were unable to produce parallel shifts in concentration-effect curves with alpha antagonists and Tuncer et al. (1985) failed to produce contractions of human umbilical arterial strips with α_2 agonist clonidine. Tuncer et al. (1985) were also unable to describe the receptor types responsible for serotonin induced contractions, though ketanserin and mianserin did produce parallel shifts in concentration-effect curves. Receptor-binding studies would help to further establish receptor types present in this tissue.

Ketanserin may provide a useful antidote for the acute toxicity of perinatal cocaine abuse. This agent has proven useful in clinical trials for the treatment of pregnancy-induced hypertension, preeclampsia-eclampsia (Rossouw et al. 1995). The potency of serotonin in the umbilical artery indicates that potentiation of serotonin responses could be very devastating to the developing fetus exposed to cocaine. Functional studies in the human umbilical artery by Tuncer et al. (1985) indicate that ketanserin may be useful in the treatment of a hypertensive situation where fetal hypoxia and placental abruption are impending.

Aims of the Study

The present study was designed to assess the toxicity of cocaine in vitro in the isolated human umbilical artery. Experiments were designed to provide information about several functional aspects of the umbilical artery itself and the impact of cocaine and its pharmacologically active metabolites in this system.

The experimental goals are as follows:

1. To determine the effect of tyramine on the isolated human umbilical artery.
These experiments would provide information about the extent of innervation in this tissue.
2. To determine the ability of cocaine and its pharmacologically active metabolites, cocaethylene, norcocaine, and benzoylecgonine, to potentiate the vasoconstrictive responses to norepinephrine and serotonin in isolated human umbilical arteries.
3. To determine the ability of serotonin₂ antagonist ketanserin to attenuate the augmentation of serotonin and norepinephrine effects by cocaine in umbilical arteries.
4. To determine the effect of methoxamine, an α_1 agonist, on umbilical arteries. This will provide information concerning the receptor types involved in the response to norepinephrine.

5. To determine if norepinephrine, serotonin, tyramine, and mianserin interact with the serotonin₂ receptors by displacement of ³H-ketanserin in isolated membrane fractions of umbilical arteries.

CHAPTER 2

MATERIALS AND METHODS

Subjects

The protocol for this study was approved by the East Tennessee State University and the Johnson City Medical Center Institutional Review Boards. Pregnant women under the care of University Physicians Practice Group - Obstetrics and Gynecology at Johnson City Medical Center were asked to participate in this study. All subjects were informed of their rights as a human subject and signed an informed consent (see Appendix A and B for informed consent and IRB approved protocol). Women with a history of perinatal drug abuse, hypertension, or pregnancy induced hypertension were excluded from the study. Umbilical cord sections were taken from the fetal end of the umbilical cord, within 15 cm of the fetus.

Contractile Studies of the Human Umbilical Artery

Tissue Preparation and Equilibration

Umbilical cords were collected from term vaginal or cesarean deliveries and immediately placed in oxygenated, modified Krebs-Henseleit buffer (see Appendix C for preparation scheme) of the following composition (mM): NaCl

(112.94), KCl (4.75), CaCl_2 (2.52), MgSO_4 (1.19), Dextrose (5.55), KH_2PO_4 (1.18), and NaHCO_3 (25.00), and stored until dissection at room temperature.

Tissues were prepared for experimentation within 2 hours of collection. Sections of artery were dissected from the cord and carefully cleared of Wharton's jelly (under buffer) using fine forceps and a FISHER Stereoscope. Sections were cut with fine surgical scissors while still under buffer to make 3mm rings of arteries. The rings were placed in individual jacketed organ baths containing modified Krebs-Henseleit buffer. The baths and the buffer were maintained at 37° C throughout the experiments and the buffer was continuously aerated with a mixture of 95% O_2 /5% CO_2 . Buffer in the organ baths was replaced every 20-30 min throughout the experiments with pre-warmed modified Krebs-Henseleit buffer.

Measurement of the Contractile Response

Umbilical artery rings were suspended horizontally in 8.2 ml organ baths by two hooks which were placed through the lumen of the vessel. The upper hook was attached to Grass model FT03C force displacement transducers (Quincy, MA) via surgical suture, and the bottom support was stabilized outside the bath with a clamp. Isometric contractile responses were recorded by a Grass Model 7D polygraph (Quincy, MA). Tissues were allowed to equilibrate for 90-120 minutes

under 2gm tension (optimal resting tension as determined empirically) then contracted every 30 min with 65mM KCl (replacing Na^+) in modified Krebs-Henseleit buffer (see Appendix D for preparation scheme) until contractions were stable to determine tissue viability and maximum standard response of each tissue.

Experiments were performed to determine the length of time tissues would maintain stable contractions with 65 mM KCl. Receptor agonists were dissolved in water and administered in 8.2 μl volumes with Eppendorf digital pipettes to yield the appropriate molar concentrations. Cumulative concentration-effect curves (van Rossum 1963) to agonists were constructed by the addition of log or 0.5 log concentration increments to the bath to yield final concentrations as indicated. A period of at least 2.5 min for measurement of maximum response was allowed after addition of each concentration. Tissues were washed at least twice after a concentration-effect curve and allowed to return to basal tension before the next concentration-effect curve was begun. Several concentration-effect curves were constructed for each agonist over a period of several hours to determine increases and decreases in sensitivity to each agonist.

Assessment of Innervation

The innervation of the human umbilical artery was assessed using tyramine, an indirect acting sympathomimetic which enters adrenergic nerve terminals and

elicits a contractile response by causing the release of endogenous norepinephrine. Tissues were exposed to 10 μ M and 100 μ M concentrations of tyramine, then washed with fresh buffer and allowed to relax. Once the tissues had relaxed to baseline tension the rings were exposed to buffer containing 10 μ M cocaine for 30 minutes then reexposed to each concentration of tyramine. A 10 μ M concentration of cocaine was used to assure complete blockade of the uptake-1 site.

Agonist Responses in the Presence and Absence of Cocaine

In order to assess the ability of cocaine to enhance responses to endogenous neurotransmitters concentration-effect curves were constructed for norepinephrine, and serotonin in the presence or absence of cocaine. Tissues were exposed to cumulative concentrations of either serotonin or norepinephrine (0.01 - 10 μ M). Tissue rings were then rinsed with fresh buffer and allowed to relax back to baseline tension. Cocaine (10 μ M) was added to the baths and after an equilibration of 30 min, the concentration-effect curves for serotonin or norepinephrine were reconstructed in the presence of cocaine. The presence or absence of cocaine was randomized and each tissue served as its own control.

Effect of Benzoecgonine, Norcocaine, and Cocaethylene on Agonist Responses

The ability of pharmacologically active metabolites of cocaine to potentiate responses to serotonin and norepinephrine (0.01 - 10 μ M) was determined and

compared to cocaine. Concentration-effect curves for norepinephrine or serotonin were constructed in log increments as described and then tissues were washed and allowed to relax to basal tension. Tissues were incubated for at least 30 min with buffer containing 10 μ M concentrations of either benzoylecgonine, norcocaine, or cocaethylene and concentration-effect curves to either serotonin or norepinephrine were reconstructed in the presence of one of these metabolites. The addition of metabolites was randomized to the control and each tissue served as its own control. Metabolite concentrations of 10 μ M were used to assure complete blockade of the uptake-1 site.

Determination of Alpha₁ Receptor Presence

In order to determine alpha₁ receptor presence in human umbilical arteries additions of methoxamine, a potent alpha₁ receptor agonist, were made. Additions were made in log increments over the concentration range 0.01 - 10 μ M. These additions were also made in the presence of 10 μ M cocaine.

Ability of Ketanserin to Attenuate Effects of Cocaine

In order to assess the ability of ketanserin to attenuate the enhanced effects of cocaine on human umbilical arteries, rings were exposed to either norepinephrine or serotonin alone, with cocaine, or with ketanserin and cocaine. Rings were exposed to cumulative concentrations (0.01 - 10 μ M in 0.5 log

increments) of either norepinephrine or serotonin. After tension equilibrated back to baseline the tissues were incubated for 30 min with cocaine (10 μ M) and the concentration-effect curves to norepinephrine and serotonin were reconstructed in the presence or absence of buffer containing ketanserin (0.03 μ M), this concentration should have produced a 30-fold shift in the concentration-effect curve for serotonin. The combinations of norepinephrine or serotonin alone, with cocaine, or with cocaine and ketanserin were randomized.

Membrane Binding Studies

Tissue Preparation and Storage

Umbilical arteries for these studies were matched to umbilical artery samples used in the ketanserin isolated tissue contractile studies. At the time of the isolated tissue studies, an additional sample of each artery, 2 cm in length, was cleaned as previously described and immediately frozen on dry ice. Tissue samples were stored at -80°C until processed for membrane binding studies.

Membrane Preparation

Arterial sections were thawed at room temperature and wet weights were determined using a digital balance. Tissue samples were cut into small (2 x 2 mm) pieces with surgical scissors under 2ml of ice-cold 250 mM sucrose solution in a

small dish, then transferred to a 25ml polystyrene tube along with an additional 5 ml of sucrose solution for a total of 7 ml and homogenized with a Tekmar *Tissuemizer* (Cincinnati, OH) at high speed for 20 sec. This homogenate was transferred to a cold 7 ml ground glass tissue grinder and manually homogenized, then transferred back to the polystyrene tube for low speed centrifugation with a Beckman Model J-6 centrifuge (Beckman Instruments, Irvine, CA) at 1086 g for 10 min at 0°C. The supernatant was diluted 10-fold with Tris buffer (50 mM, pH 7.7), and centrifuged with a Beckman Model L5-65B ultracentrifuge (Beckman Instruments, Irvine, CA) at 50,000 g for 15 min at 0°C. The supernatant was discarded and the pellet was resuspended in 12.5 ml of fresh Tris buffer. The membrane suspension was frozen at -80°C until the binding experiments were conducted (see Figure 2 on the following page for a diagram of the preparation scheme).

Binding Assay Procedure

Protein Assay. Aliquots (100 µl) of the membrane preparation samples in Tris buffer were assayed for protein content with bovine serum albumin as the protein standard using Bradford (1976) microtiter plate protocol, see Appendix E for protocol.

Competitive Binding Assay. The binding characteristics of unlabeled ketanserin, mianserin, serotonin, or tyramine (0.001 μ M - 100 μ M), and norepinephrine (10 μ M) were determined by competitive binding of these agents with 3 H-ketanserin. The assay was performed using the *Millipore Multi-Screen Filtration System*, this 96-well plate contained individual 1.2 μ m glass fiber type C filters. The assay was carried out in these wells on ice with 0.7 - 1.0 μ g protein per 100 μ l, 3 nM of 3 H-ketanserin (50 μ l), and 100 μ l of competitive drug for a total volume of 250 μ l in each. Competitive binding assays for serotonin, norepinephrine, and tyramine also included 10 μ M pargyline and 0.1% ascorbic acid to prevent their metabolism during incubation. All buffers and drug dilutions were kept ice-cold and additions were made on ice. The plates were incubated in a Reciprocal Shaking Bath Model 25 (Precision Scientific, Chicago, IL) for 20 min at 37° C, pH 7.4 with agitation (60 cycles per min).

At the end of the incubation period, the plates were quickly removed from the incubator and placed in the Millipore vacuum manifold. Contents of the wells were vacuum filtered and washed once with 250 μ l Tris buffer (room temperature). Each filter was removed from the plate and placed in a 7 ml scintillation vial with 4 ml of *CytoScint* (Fisher Biotech, Pittsburgh, PA) scintillation cocktail. Vials were counted for 10 min each in a Wallac model 1409 liquid scintillation counter (Gaithersburg, MD).

Calculations and Statistics

Contractile Studies

Data are represented by the mean \pm standard error of the mean (S.E.M.) responses expressed as a percent of the paired, KCl maximum, for 5 subjects. Data representation by this method decreased the variation between subjects and allowed for more rigorous comparisons. Concentration-effect curves were compared by several different methods. Comparisons were always made for each dose of agonist in the presence and absence of cocaine using paired Student's t-tests. Cumulative response was further analyzed by calculation of the area under the concentration-effect curve for each tissue in the presence and absence of cocaine using the trapezoidal rule (Wagner 1979). The area under the concentration-effect curve was defined as:

$$\sum 1/2(C_1 + C_2)(R_2 - R_1),$$

where C = molar concentration of drug and R = % KCl Max. EC_{50} and EC_{15} values, the mean effective concentrations to produce either 50 or 15% of the maximum response to KCl, were calculated from log interpolation of each concentration-effect curve. Dissociation constants (K_B) for norepinephrine and serotonin were calculated using the equation from Kenakin et al. (1992),

$$\log [Dose\ Ratio-1] = \log [Antagonist] - \log K_B,$$

where the dose ratio is the EC_{50} after antagonist addition / EC_{50} before antagonist addition and [Antagonist] is the concentration of ketanserin (0.03 μ M).

Levels of $p \leq 0.05$ were accepted as statistically significant differences between groups compared. Statistical comparisons were made on a *GATEWAY 2000* Personal Computer P5-166 XL and *PROSTAT* statistics package version 1.50 and *PSI-PLOT* graphics package version 5.0, (Poly Software International, Salt Lake City, UT). Graphs were created using Graphpad *PRISM* version 2.01 graphics package, (Graphpad Software, Inc., San Diego, CA).

3 H-Ketanserin Binding Studies

Data obtained from the radioligand binding assays were converted from disintegrations per minute to picomoles of binding per milligram of protein using the Graphpad *PRISM* graphics package on a personal computer as previously described. The saturation binding curve for 3 H-ketanserin was determined from the unlabeled ketanserin displacement curve. These values were then used to determine the binding maximum (B_{max}) and dissociation constant (K_d) of 3 H-ketanserin in the protein samples obtained from human umbilical arteries matched to ketanserin isolated tissue studies. Using Graphpad *PRISM* nonlinear regression analysis, the data were fit to the equation:

$$Total\ Binding = \frac{B_{max} [Ligand]}{K_d + [Ligand]} + N [Ligand],$$

where $N * [Ligand]$ represents the nonspecific binding of the ligand. The B_{max} represents the maximum number of specific binding sites in pmoles per mg protein and K_d represents the dissociation constant in nM of 3H -ketanserin for the binding site.

The data from the competitive binding studies were transformed to percent of control binding for each of the displacing ligands. These values were then fit to a one or two site binding equation based on the calculated best fit using the Graphpad *PRISM* curve fitting program and the sequential F-test.

One site competition equation:

$$y = bottom + (top - bottom) / (1 + 10^{(x - log EC50)})$$

Two site competition equation:

$$y = (bottom + top) * fraction\ 1 / (1 + 10^{(x - log EC50_1)}) + \\ (bottom + top) * (1 - fraction1) / (1 + 10^{(x - log EC50_2)})$$

Materials and Chemicals

Contractile Studies

(-)-Norepinephrine bitartrate, tyramine hydrochloride, 5-hydroxytryptamine hydrochloride, methoxamine hydrochloride, and dopamine hydrochloride, were purchased from Sigma Chemical Co., (St. Louis, MO). Norcocaine hydrochloride, cocaethylene hydrochloride, benzyolecgonine, cocaine hydrochloride, and ketanserin tartrate were purchased from Research Biochemicals International, (Natick, MA). All drugs were dissolved and diluted with ultra pure deionized distilled water (18 mOhm). Chemicals for modified Krebs-Henseleit buffer were purchased from Fisher Scientific (Pittsburgh, PA).

Binding Studies

Ketanserin hydrochloride, [ethylene-³H]-(R41 468), specific activity range 60-90 Ci/mmol, > 97% radiochemical purity was obtained from New England Nuclear Life Science Products, Inc. (Boston, MA). Unlabeled competitive binding agents norepinephrine bitartrate, 5-hydroxytryptamine hydrochloride, and tyramine were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved and diluted in Tris buffer, pH 7.7. Ketanserin tartrate, and mianserin hydrochloride were purchased from Research Biochemicals International, (Natick, MA), dissolved in 0.1 N HCl and diluted with Tris buffer. L-ascorbic acid and

biological grade tris, and *CYTOSCINT* scintillation cocktail was obtained from Fisher Biotech, (Fair Lawn, NJ). Pargyline hydrochloride was purchased from Regis Chemical Co.

CHAPTER 3

RESULTS

Contractile Studies in the Human Umbilical Artery

Normal Contractile Function

The exposure of umbilical artery rings to 65 mM KCl produced a rapid increase in contractile force that reached a maximum within 30-60 seconds and was sustained at a steady tonic phase for 2-3 min (Figure 3). Responses to KCl were stable over the course of the experimental time frame of 5-6 hours. Tyramine produced a similar response also shown in Figure 3, contractions to tyramine were also stable over a 2-3 hour period.

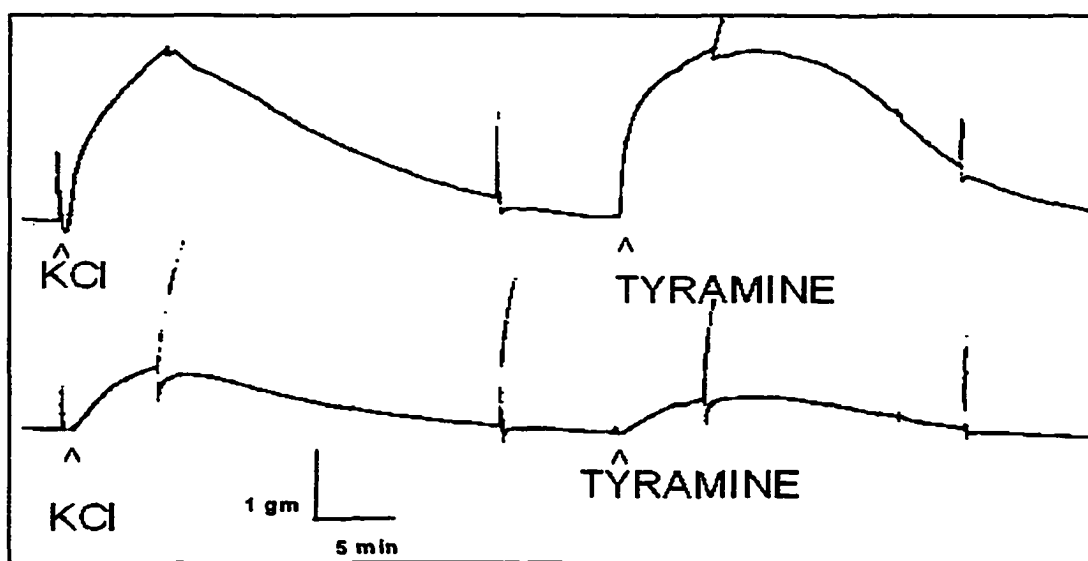


Figure 3. Typical isometric responses to 65mM KCl and tyramine.

Isolated umbilical artery rings were used to produce concentration-effect curves for norepinephrine and serotonin over the concentration range 0.01 - 10 μ M, these concentration-effect curves were repeatable and remained stable for up to 5 hours.

Assessment of Innervation

In order to assess the presence of innervation in the isolated umbilical artery tyramine was added to the preparation in 10 and 100 μ M concentrations as previously described in the Materials and Methods section. Tissues were also exposed to tyramine in the presence of 10 μ M cocaine. Tyramine (10 μ M) produced a slight vasoconstrictive response. Addition of cocaine (10 μ M) did not significantly potentiate this response (Figure 4). The mean vasoconstrictive response to tyramine (100 μ M) was significantly increased by 257% ($p < 0.05$), in the presence of cocaine (10 μ M) in comparison to tyramine alone at 100 μ M (Figure 4).

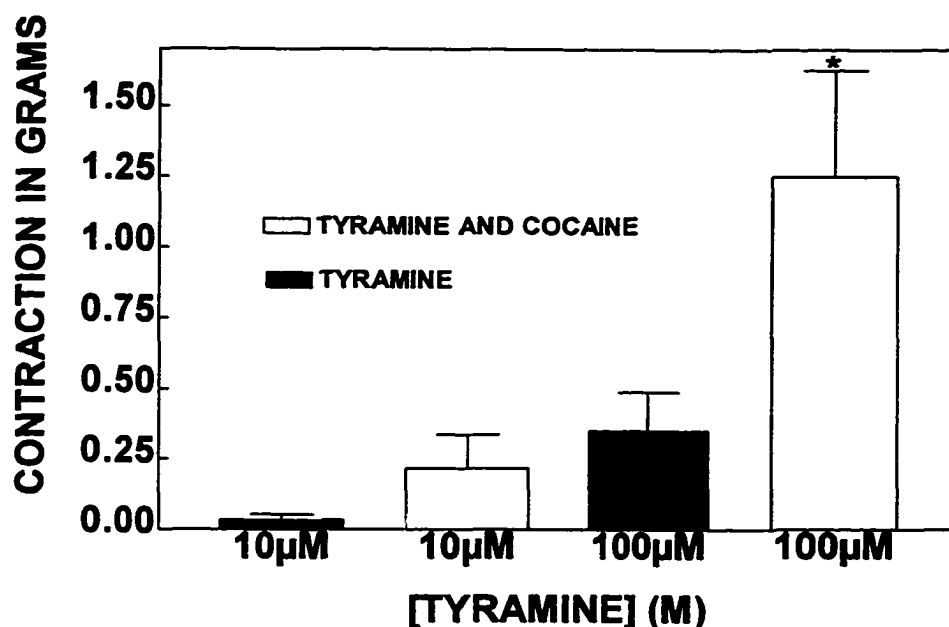


Figure 4. Effect of cocaine (10 μ M) on the vasoconstrictive response to tyramine. Data are presented as mean \pm S.E.M. for paired tissue comparisons, $n=6$, * $p \leq 0.05$.

Agonist Responses in the Presence of Cocaine

Effect of Cocaine on Serotonin Responses. The dose-response curves for serotonin in the presence and absence of cocaine (10 μ M) are presented in Figure 5. Cocaine produced a potentiation of the vasoconstrictive response to serotonin. In the presence of cocaine, the mean vasoconstrictive effect of serotonin from 0.1 μ M to 10 μ M was potentiated by 28% ($p < 0.05$). In the presence of cocaine, the tissue was more sensitive to serotonin. The area under the concentration-effect curve for serotonin in the presence of cocaine was significantly greater ($p < 0.05$) than the area for serotonin alone (Table 1).

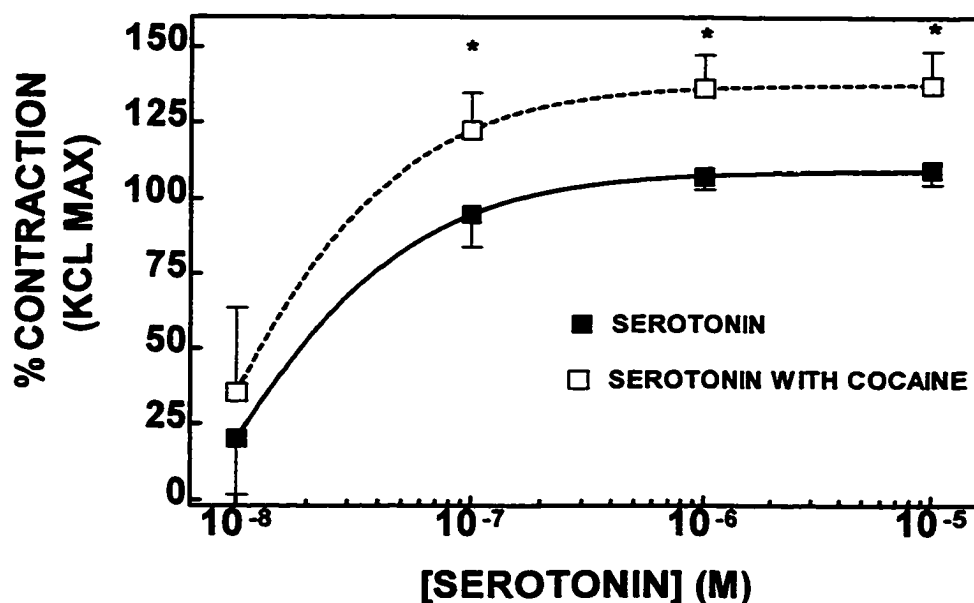


Figure 5. Effect of cocaine (10 μ M) on the vasoconstrictive response to serotonin. Data are presented as the mean \pm S.E.M of paired tissue comparisons, $n=6$, * $p \leq 0.05$.

TABLE 1.

Area under the cumulative concentration-effect

curve⁺ for serotonin and norepinephrine in the presence and absence of cocaine.

Neurotransmitter	Neurotransmitter Alone	Neurotransmitter with Cocaine
Norepinephrine	176.19 (51.30)	614.87* (203.49)
Serotonin	1071.65 (42.58)	1355.50* (111.82)

Data is the mean (S.E.M.) of 6 paired observations.

* indicates statistical significance ($p < 0.05$).

⁺ Area under the cumulative concentration effect curve is defined as

$\Sigma 1/2(C_1 + C_2)(R_2 - R_1)$ where C = concentration of neurotransmitter and

R = %contraction (KCl max)/ μ M concentration.

Effect of Cocaine on Norepinephrine Responses. The concentration-effect curves produced by norepinephrine in this model were reproducible and the contractile response was induced in a dose-dependent manner. The concentration-effect curves for norepinephrine in the presence and absence of cocaine (10 μ M) are presented in Figure 6 . Cocaine potentiated the sensitivity to norepinephrine by about 25% at lower doses and by 64% at the maximum response to norepinephrine (10 μ M) significantly, ($p < 0.05$). The area under the concentration-effect curve for norepinephrine in the presence of cocaine was significantly greater than the area for norepinephrine alone ($p < 0.05$), (Table 1). Cocaine produced a 60-fold, leftward shift in the concentration-effect curve. This indicates greater sensitivity of the umbilical artery to norepinephrine in the presence of cocaine.

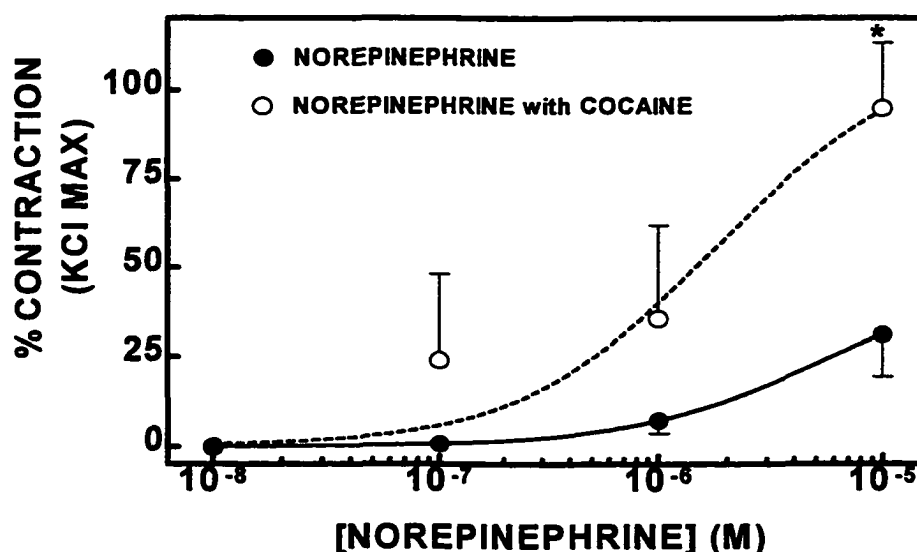


Figure 6. Effect of cocaine (10 μ M) on the vasoconstrictive response to norepinephrine. Data are presented as the mean \pm S.E.M. of paired tissue comparisons, $n=6$, * $p < 0.05$.

Agonist Responses in the Presence of Benzoylecgonine

Effect of Benzoylecgonine on Serotonin Responses. The concentration-effect curves for serotonin alone and in the presence of benzoylecgonine are presented in Figure 7. Benzoylecgonine produced a decrease in the contractile response of the umbilical artery to serotonin, this effect was significant at 1 and 10 μ M where the responses were reduced by 36 and 30% respectively. The shift of the concentration-effect curve to the right indicates a decrease in sensitivity to serotonin in the presence of benzoylecgonine. The area under the cumulative-concentration effect curve values for serotonin in the presence of benzoylecgonine were significantly less than for serotonin alone (Table 2).

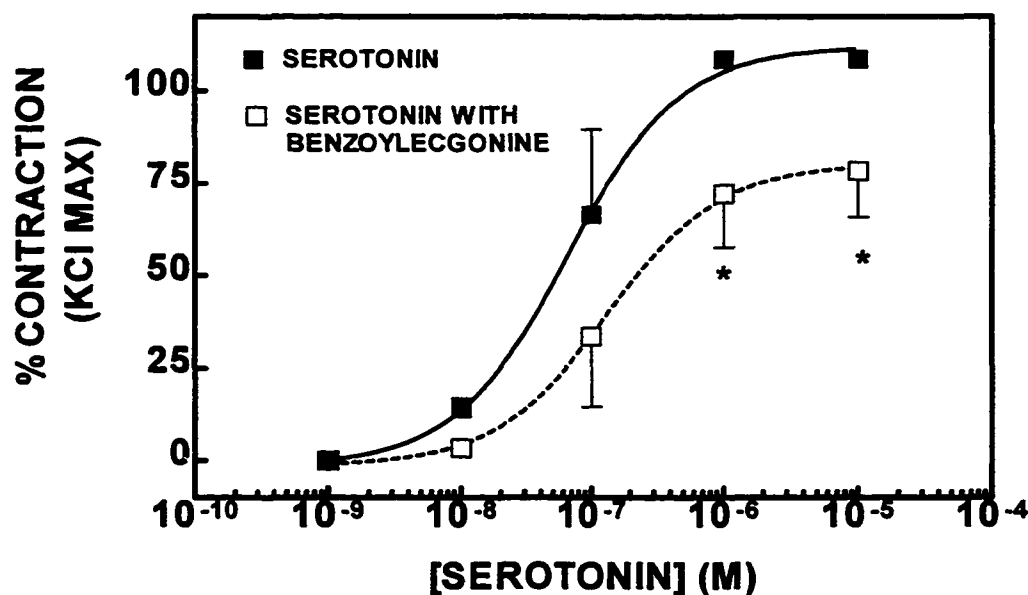


Figure 7. Effect of benzoylecgonine (10 μ M) on the vasoconstrictive response to serotonin. Data are presented as the mean \pm S.E.M. of paired tissue responses, $n=4$, * $p \leq 0.05$.

TABLE 2.

Area under the cumulative concentration-effect curve* for serotonin and
norepinephrine in the presence and absence of benzoylecgonine

Neurotransmitter	Neurotransmitter alone	Neurotransmitter with benzoylecgonine
Norepinephrine	404.25 (162.13)	452.25 (196.67)
Serotonin	1057.5 (20.3)	714.0* (124.5)

Data is the mean (S.E.M.) of 4 paired observations

* indicates statistical significance ($p < 0.05$).

+ Area under the cumulative concentration effect curve is defined as

$\Sigma 1/2(C_1 + C_2)(R_2 - R_1)$ where C = concentration of neurotransmitter

R = % contraction (KCl max)/ μ M concentration.

Effect of Benzoylecgonine on Norepinephrine Responses. The concentration-effect curves for norepinephrine in the presence and absence of benzoylecgonine are presented in Figure 8. Benzoylecgonine produced no significant differences on the responses of the isolated umbilical artery to norepinephrine. Benzoylecgonine did shift the EC₁₅ for norepinephrine to the right 4.5 fold, indicating decreased sensitivity of the tissue to norepinephrine in the presence of benzoylecgonine.

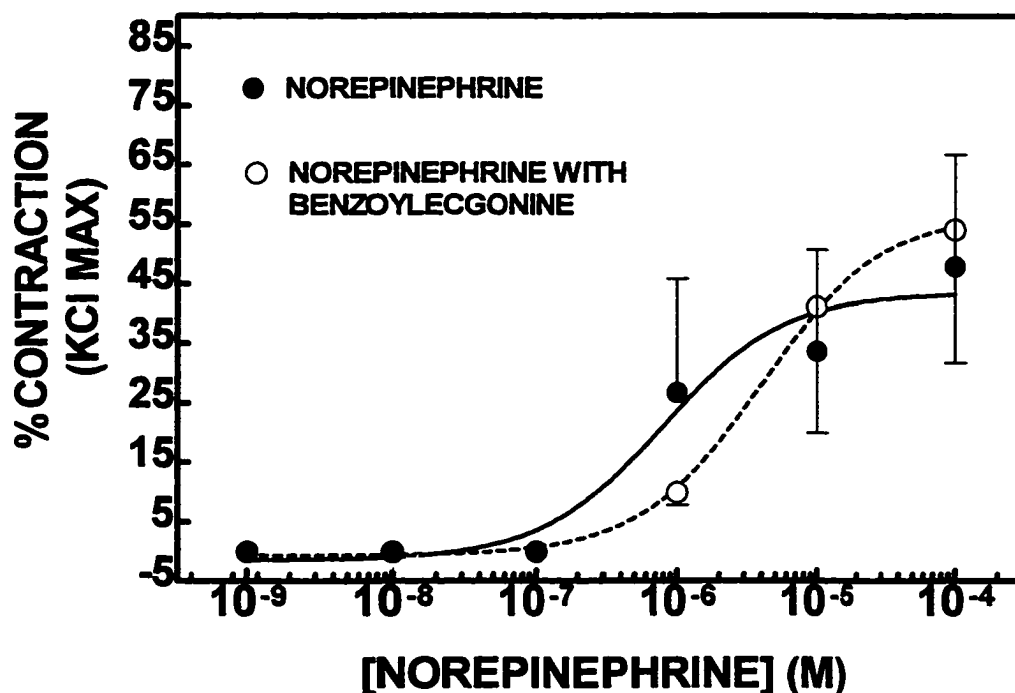


Figure 8. Effect of benzoylecgonine (10 μ M) on the vasoconstrictive response to norepinephrine. Data are presented as paired tissue comparisons, mean \pm S.E.M., n=4.

Agonist Responses in the Presence of Norcocaine

Effect of Norcocaine on Serotonin Responses. Concentration-effect curves for serotonin in the presence and absence of norcocaine (10 μ M) are presented in Figure 9. Serotonin responses in the presence of norcocaine were not significantly different from control responses. Norcocaine did produce a slight leftward shift in the concentration-effect curve for serotonin and a slight potentiation of the maximum response (22%).

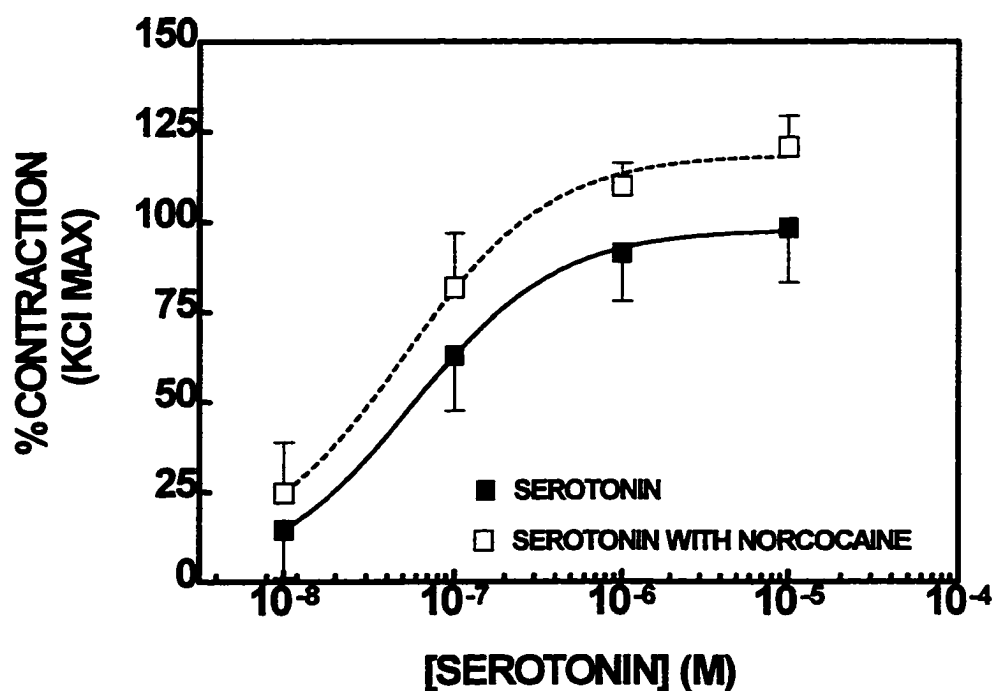


Figure 9. Effect of norcocaine (10 μ M) on the vasoconstrictive response to serotonin. Data are presented as paired tissue comparisons, mean \pm S.E.M. n=6.

Effect of Norcocaine on Norepinephrine Responses. The concentration-effect curves for norepinephrine in the presence and absence of norcocaine (10 μ M) are presented in Figure 10. Norcocaine significantly potentiated the maximum response to norepinephrine by 54%, $p < 0.05$. EC_{50} values and area under the concentration-effect curve values for norepinephrine alone and norepinephrine in the presence of norcocaine were not significantly different, due to large variations among tissue responses.

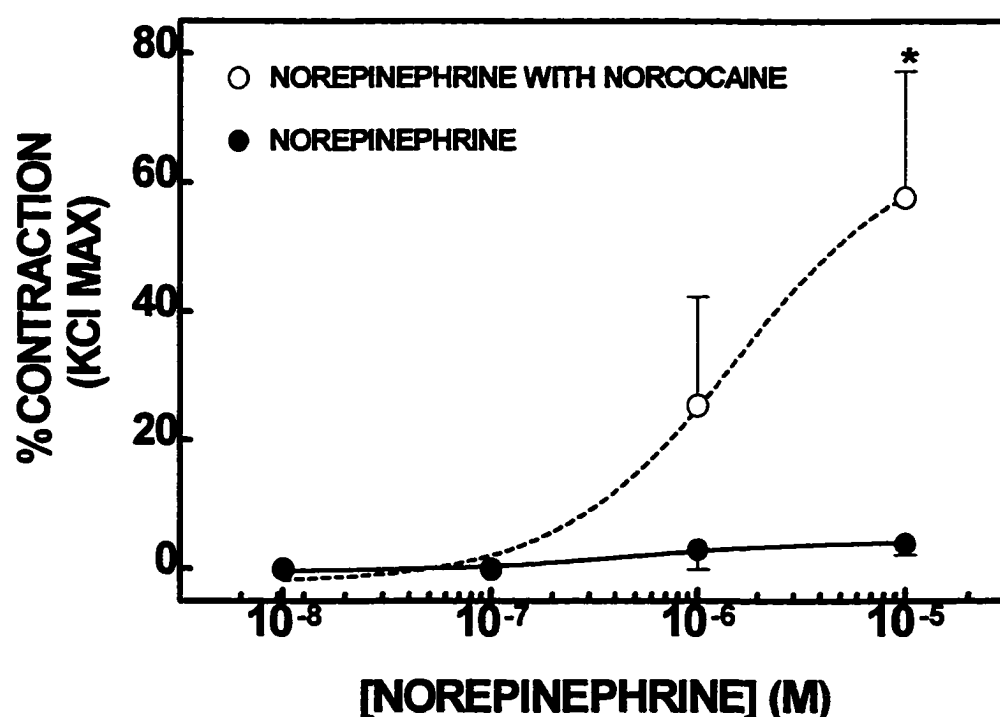


Figure 10. Effect of norcocaine (10 μ M) on the vasoconstrictive response to norepinephrine. Data are presented as the mean \pm S.E.M. of paired tissue comparisons, $n=6$, $* \leq p 0.05$.

Agonist Responses in the Presence of Cocaethylene

Effect of Cocaethylene on Serotonin Responses. The responses of the isolated human umbilical artery to serotonin in the presence and absence of cocaethylene are presented in Figure 11. Cocaethylene did not potentiate the concentration-effect curve to serotonin significantly. An increase in maximum responses of 23% indicates increased sensitivity to serotonin of the tissue in the presence of cocaethylene.

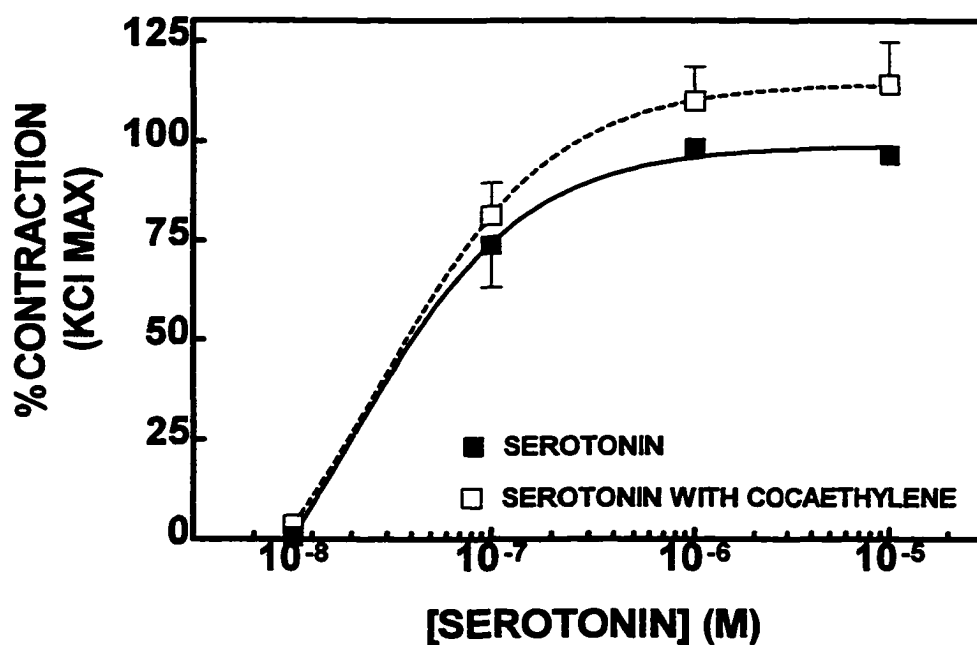


Figure 11. Effect of cocaethylene (10 μ M) on the vasoconstrictive response to serotonin. Data are presented as the mean \pm S.E.M. of paired tissue comparisons, n=7.

Effect of Cocaethylene on Norepinephrine Responses. The concentration-effect curves for norepinephrine alone and in the presence of cocaethylene are presented in Figure 12. Cocaethylene potentiated the maximum response to norepinephrine by 44%. The leftward shift of the concentration-effect curve indicates greater sensitivity of the tissue to norepinephrine in the presence of cocaethylene. There were no significant differences between concentration-effect curves despite the obvious effect of cocaethylene, this is probably due to large variations among tissue responses.

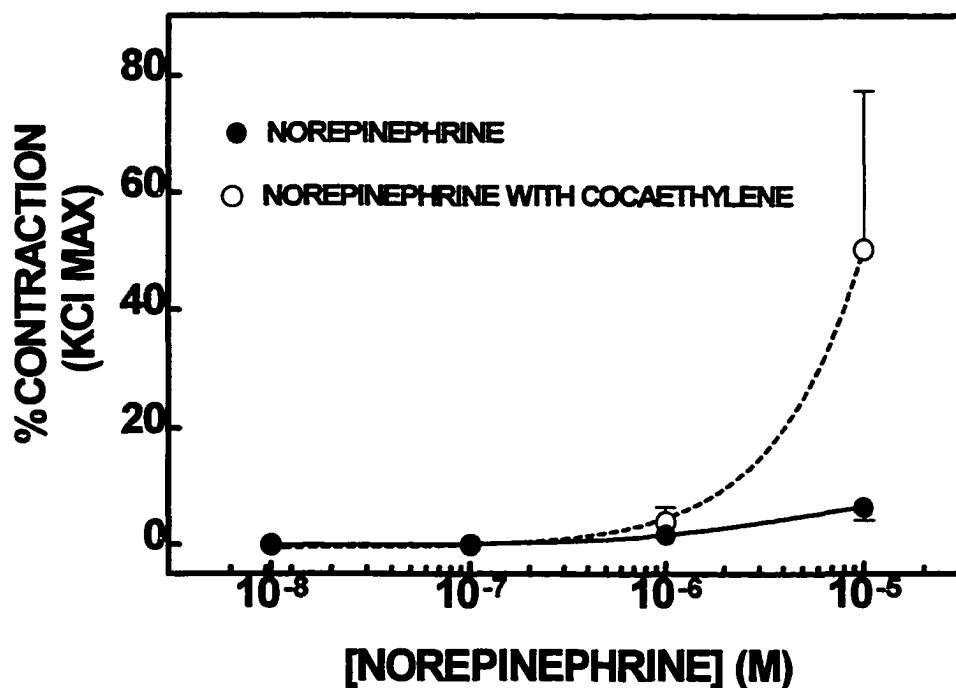


Figure 12. Effect of cocaethylene (10 μ M) on the vasoconstrictive response to norepinephrine. Data are presented as the mean \pm S.E.M. of paired tissue comparisons, $n=7$.

Comparison of Potency Between Cocaine and Metabolites

Area under the concentration-effect curve values were determined for serotonin and norepinephrine in the presence or absence of cocaine and its metabolites. The differences in the concentration-effect curves between the presence or absence of cocaine or its metabolites for each neurotransmitter were calculated and used to perform a multiple comparisons analysis of variance between effects of cocaine and its metabolites. The mean values for the area under the concentration-effect curve differences for serotonin in the presence or absence of cocaine or metabolites are presented in Table 3. The mean values for area under the concentration-effect curve differences for norepinephrine in the presence and absence of cocaine or metabolites are presented in Table 4. The effect for serotonin with cocaine, norcocaine, or cocaethylene was significantly greater than that of serotonin with benzoylecgonine. The order of potency for cocaine and its metabolites based on the greatest to the least effect between the presence and absence of cocaine or metabolite in response to serotonin and norepinephrine is:

cocaine≥norcocaine≥cocaethylene>>>>benzoylecgonine.

TABLE 3.

Cummulative effects⁺ for serotonin in the
presence or absence of cocaine or metabolites.

Serotonin / Cocaine	Serotonin / Benzoylecgonine	Serotonin / Norcocaine	Serotonin / Cocaethylene
283.80 (109.49)	-351.00* (139.35)	201.85 (103.18)	141.28 (89.62)

* $p \leq 0.05$

+Data is the mean, % contraction (KCl MAX)/ μ M concentration, (S.E.M.) of 4-7 paired observations.

TABLE 4.

Cummulative effects⁺ for norepinephrine in the presence of cocaine or metabolites.

Norepinephrine/ Cocaine	Norepinephrine/ Benzoylecgonine	Norepinephrine/ Norcocaine	Norepinephrine/ Cocaethylene
439.52 (167.26)	48.00 (38.42)	320.46 (156.28)	262.31 (125.88)

+Data is the mean, % contraction (KCl MAX)/ μ M concentration, (S.E.M.) of 4-7 paired observations.

Determination of α_1 Receptor Presence

Additions of methoxamine, a potent α_1 agonist, were made to tissue bath preparations. The addition of increasing concentrations of methoxamine (0.01 - 10 μ M) produced no effect on the human umbilical artery. There was no change from baseline tension over the entire concentration range.

Effect of Ketanserin on Cocaine Potentiation of Serotonin Responses

The concentration-effect curves for serotonin alone, serotonin with cocaine, and serotonin with cocaine and ketanserin are presented in Figure 13. Calculated EC₅₀'s for each drug combination are given in Table 5. Cocaine potentiated the response to serotonin as shown by the leftward shift of the concentration-effect curve to serotonin in the presence of 10 μ M cocaine. Ketanserin (0.03 μ M) was able to attenuate the enhanced vasoconstriction produced by cocaine. Ketanserin significantly increased the EC₅₀ for serotonin with cocaine, shifting the EC₅₀ to the right 10-fold ($p < 0.05$). The calculated pK_B value ($\log [\text{dose ratio} - 1] = \log [\text{antagonist}] - \log K_B$) for serotonin with cocaine was 8.30. Ketanserin was also able to significantly depress the response to serotonin at 0.03 μ M and 0.1 μ M by 87% and 102% respectively ($p < 0.05$) in the presence of cocaine (10 μ M).

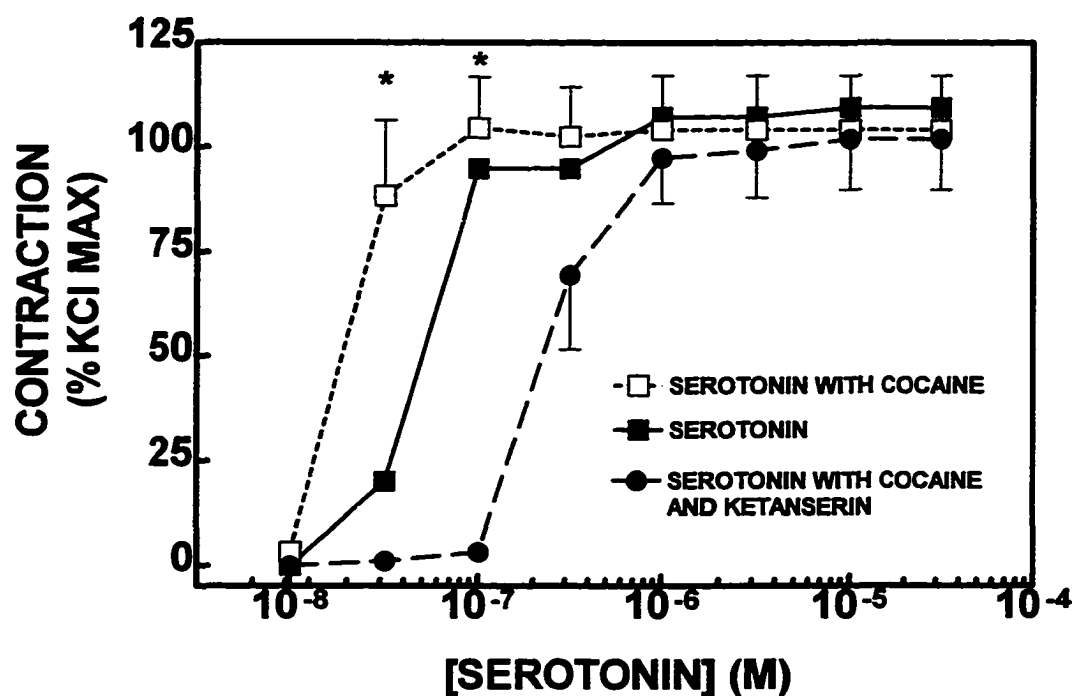


Figure 13. Effect of ketanserin (0.03 μ M) on the potentiation of serotonin by cocaine (10 μ M). Data are presented as the mean \pm S.E.M. of paired tissue comparisons, $n=5$, * $p \leq 0.05$.

TABLE 5.

EC50 values for serotonin alone, with cocaine and with cocaine and ketanserin.

Serotonin	0.049 \pm .006 μ M
Serotonin / Cocaine	0.022 \pm .005 μ M
Serotonin / Cocaine / Ketanserin	0.32 \pm .09 μ M *

Data is the mean \pm the S.E.M.

* - Significantly different from serotonin with cocaine, $p < 0.05$

Effect of Ketanserin on Cocaine Potentiation of Norepinephrine Responses

The concentration-effect curves for norepinephrine alone, norepinephrine with cocaine, and norepinephrine with cocaine and ketanserin are presented in Figure 14. EC₁₅ values, were calculated and used for comparison because this is the responses at which parallel shifts in the concentration-effect curves for norepinephrine can best be compared. The EC₁₅ values for each drug combination are given in Table 6. Cocaine was able to potentiate the concentration-effect curve to norepinephrine as shown by the 205-fold leftward shift of the EC₁₅ in the presence of 10 μ M cocaine. The potentiation of norepinephrine by cocaine was completely attenuated by ketanserin (0.03 μ M) in this tissue. Ketanserin produced a 205-fold rightward shift of the EC₁₅ to norepinephrine in the presence of cocaine. The calculated pK_B value for norepinephrine with cocaine was 9.17. Ketanserin significantly depressed the maximum response to norepinephrine by 54% at 1 μ M ($p < 0.05$) in the presence of 10 μ M cocaine.

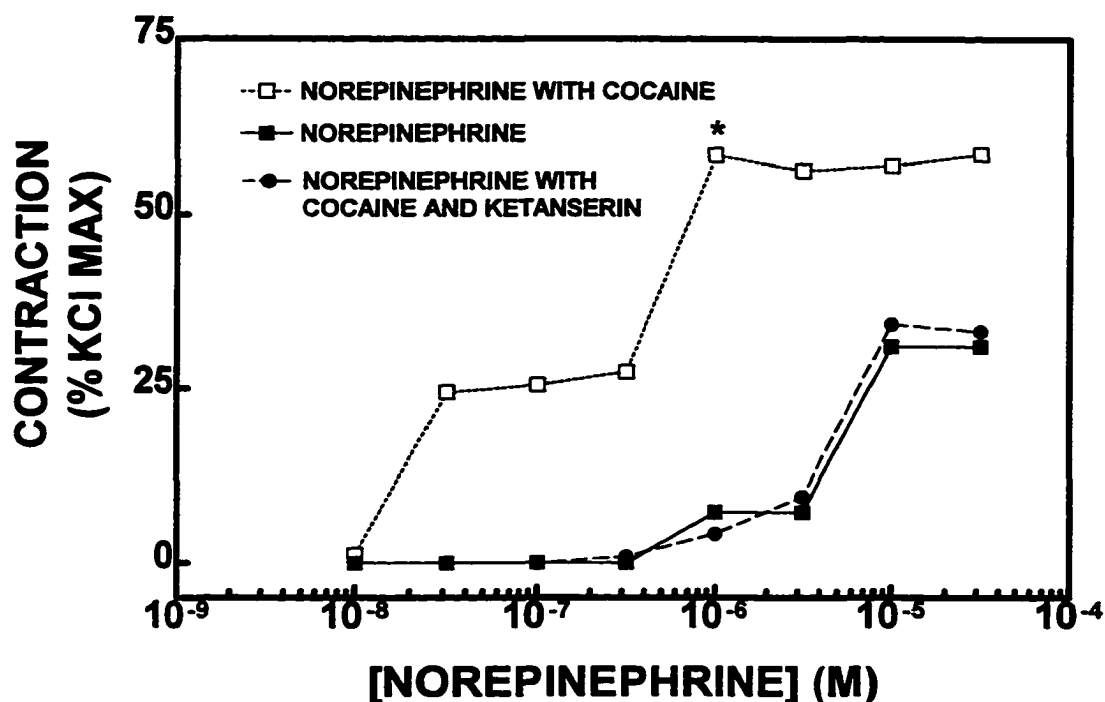


Figure 14. Effect of ketanserin (0.03 μM) on the potentiation of norepinephrine by cocaine (10 μM). Data are presented as the mean of paired tissue comparisons, $n=5$, $*p \leq 0.05$.

TABLE 6.

EC15 values for norepinephrine alone, with cocaine, and with cocaine and ketanserin.

Norepinephrine/Cocaine	$0.79 \pm 0.05 \mu\text{M}$
Norepinephrine/Cocaine/Ketanserin	$3.6^* \pm 1.5 \mu\text{M}$

Data is the mean (standard deviation).

* - Significantly different from norepinephrine with cocaine, $p < 0.05$

³H-Ketanserin Membrane Binding Studies

³H-Ketanserin Saturation

Umbilical arteries were homogenized and prepared as described in the Materials and Methods. The membrane fraction was assayed for serotonin_{2a} and serotonin_{2c} binding characteristics. The binding maximum (B_{\max}) and the dissociation constant (K_D) for ³H-Ketanserin was calculated using Graphpad *PRISM* nonlinear regression analysis. The binding characteristics of ³H-Ketanserin fit best to a one site binding equation as determined by the sequential F-test. B_{\max} was 14.610 nmol/mg, K_D was 241.6 nM, and the non-specific binding was 112.204 nmol/mg. The saturation curve for ³H-Ketanserin is presented in Figure 15.

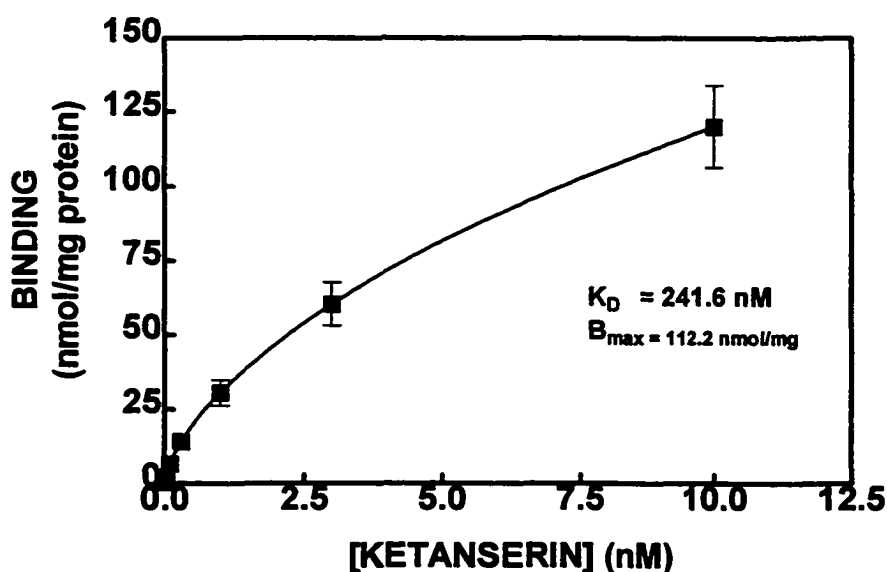


Figure 15. ³H-ketanserin saturation binding characteristics. Data are presented as the mean \pm S.E.M., $n=6$.

Displacement of ^3H -Ketanserin by Mianserin

^3H -ketanserin was competitively displaced by mianserin, a serotonin_{2a,b,&c} antagonist. The K_D for mianserin was $0.29 \pm .083 \mu\text{M}$, the displacement curve fit best to the one-site competition equation according to the sequential F-test, $F = 5.746$ and $p = 0.01629$. The displacement curve for mianserin presented in Figure 16.

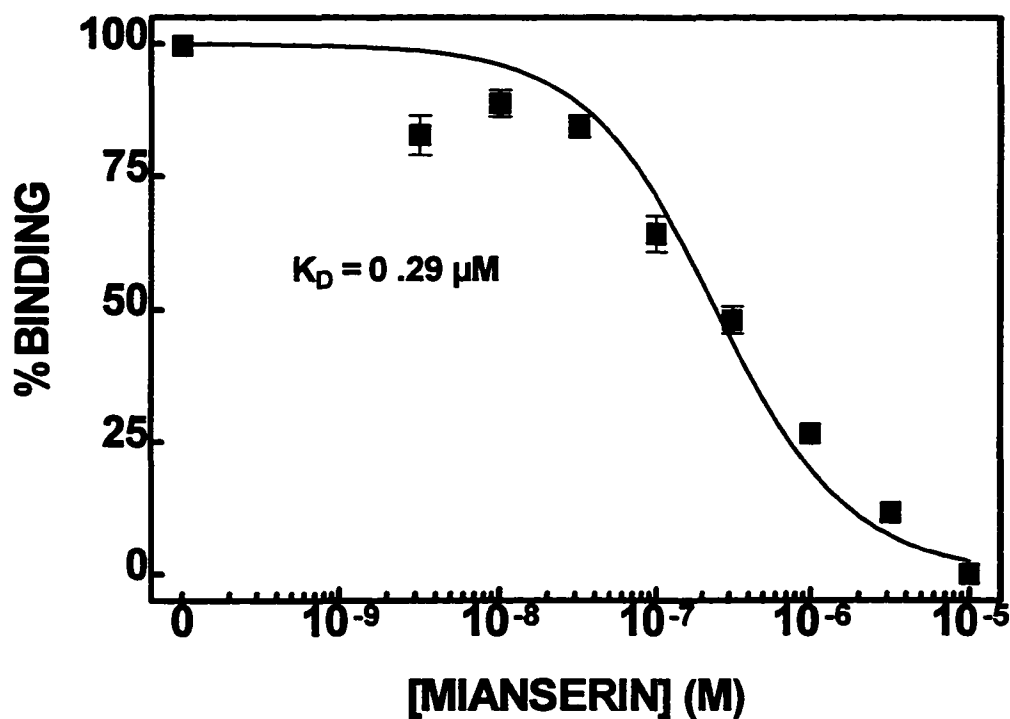


Figure 16. Mianserin displacement of ^3H -ketanserin (3 nM). Data are presented as the mean \pm S.E.M., $n=6$.

Serotonin Displacement of ^3H -Ketanserin

Serotonin competitively displaced ^3H -ketanserin. The displacement curve for serotonin fit best to the two-site competition equation, $F = 6.2$ and $p = 0.04$ with a K_D 2.8 ± 1.9 nM for one site and a K_D 2.7 ± 0.72 μM for the second site.

The displacement curve for serotonin is presented in Figure 17.

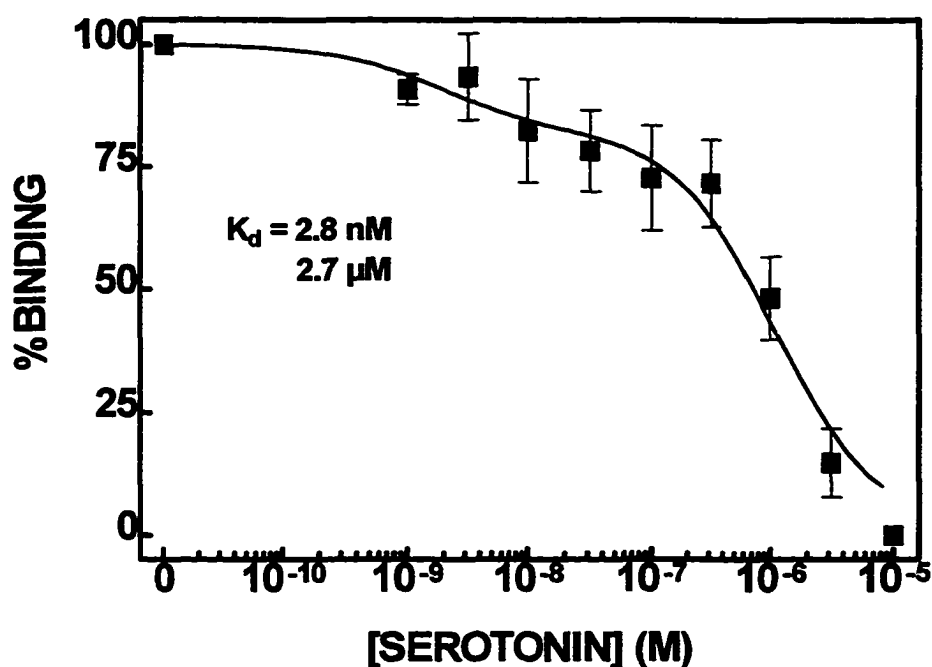


Figure 17. Serotonin displacement of ^3H -ketanserin (3 nM). Data are presented as the mean \pm S.E.M., $n=6$.

Tyramine Displacement of ^3H -Ketanserin

Tyramine was able to displace ^3H -ketanserin at two sites as determined by the sequential F-test, $F = 21.56$ and $p = 0.011$. The K_D for one site was 7.88 ± 7.82 nM and the K_D for the second site was 36.24 ± 32.44 μM . The displacement curve for tyramine is presented in Figure 18.

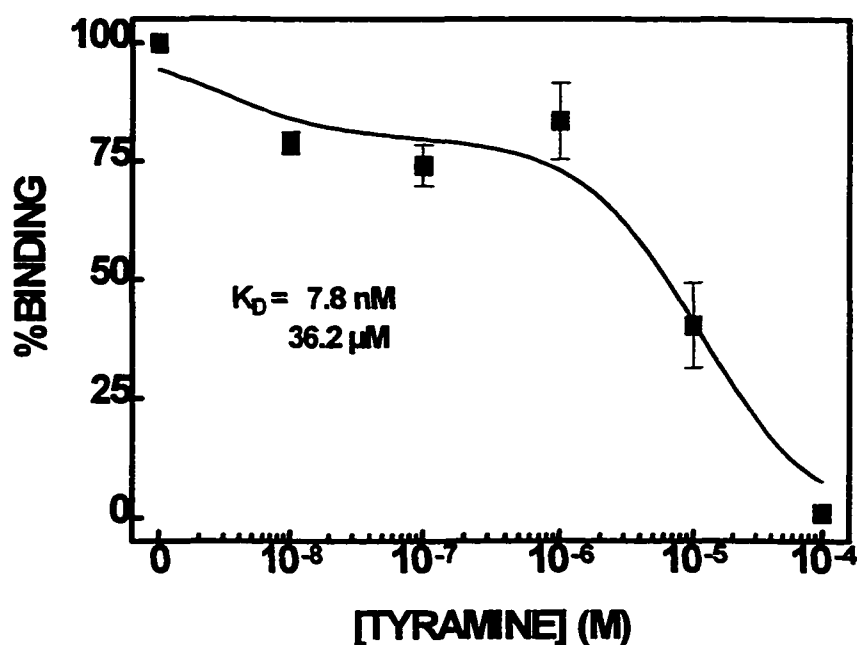


Figure 18. Tyramine displacement of ^3H -ketanserin (3 nM). Data are presented as the mean \pm S.E.M., $n=6$.

Norepinephrine Displacement of ^3H -Ketanserin

Norepinephrine produced minimal displacement of ^3H -ketanserin, only 4% of total binding was displaced (Figure 19). Even at a concentration of 10 μM norepinephrine there was no significance difference from the control.

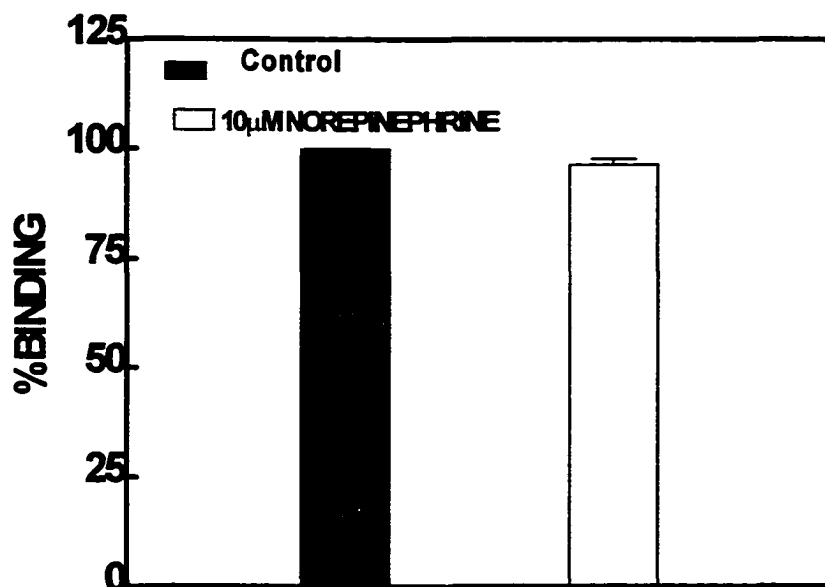


Figure 19. Norepinephrine displacement of ^3H -ketanserin (3 nM). Data are presented as the mean \pm S.E.M., n=6.

CHAPTER 4

DISCUSSION

The in vitro toxicity of cocaine and its metabolites and the antidotal potential for ketanserin in human umbilical artery toxicity produced by cocaine were examined in this study. Cocaine abuse has been implicated in a number of perinatal complications presumably arising from the vasoconstrictive actions of cocaine such as placental abruption and preterm labor. Studies conducted in the isolated ovine (Dyer 1970) and guinea pig (Nair and Dyer 1974) umbilical arteries have provided evidence that cocaine may potentiate serotonin and norepinephrine constriction of umbilical arteries. Several cocaine metabolites, benzoylecgonine, norcocaine, and cocaethylene seem to possess pharmacological activity similar to cocaine itself, in that all three produced vasoconstriction of fetal cerebral porcine arterioles (Kurth et al. 1993). Tuncer et al. (1985) reported that ketanserin and mianserin antagonized serotonin induced contractions of human umbilical artery strips, and ketanserin has been touted as a safer alternative to hydralazine in perinatal hypertension (Rossouw et al. 1995).

Innervation of the human umbilical artery was investigated using tyramine, an indirect acting sympathomimetic, in an isolated tissue model. Concentrations of tyramine were added to the baths to produce vasoconstriction which should be

abolished by the addition of cocaine. The ability of cocaine to produce enhanced contractile responses to serotonin and norepinephrine in the isolated umbilical artery ring model was analyzed. Cocaine metabolites, benzoylecgonine, norcocaine, and cocaethylene were also examined for their ability to enhance responses to serotonin and norepinephrine. The serotonin₂ antagonist ketanserin was used to delineate the question of receptor types involved in cocaine potentiation of vasoconstrictive responses. Membrane binding studies were conducted using ³H-ketanserin to determine the displacement characteristics of mianserin, serotonin, tyramine, and norepinephrine at serotonin₂ receptors in the smooth muscle of the umbilical artery.

Innervation of the Isolated Human Umbilical Artery

The innervation of the umbilical vasculature has been a topic of controversy for several decades. Even as recently as 1990 the conflict continued; no evidence of innervation in the umbilical vasculature was initially demonstrated using immunohistological and histochemical techniques (Fox and Khong 1990), yet in another study the presence of adrenergic and cholinergic nerve fibers were revealed in umbilical arteries at the fetal end of the human umbilical cord using histochemical techniques (Kawano and Mori 1990). The possibility of adrenergic

innervation in human umbilical artery suggests a role for cocaine in potentiating vasoconstriction produced by norepinephrine and serotonin.

Tyramine is an indirect-acting sympathomimetic substance which produces a pressor response in other tissues by producing norepinephrine release from presynaptic nerve terminals (Figure 20). The vasoconstrictive effect of tyramine in other tissues is abolished or inhibited by cocaine's blockade of the presynaptic uptake of tyramine.

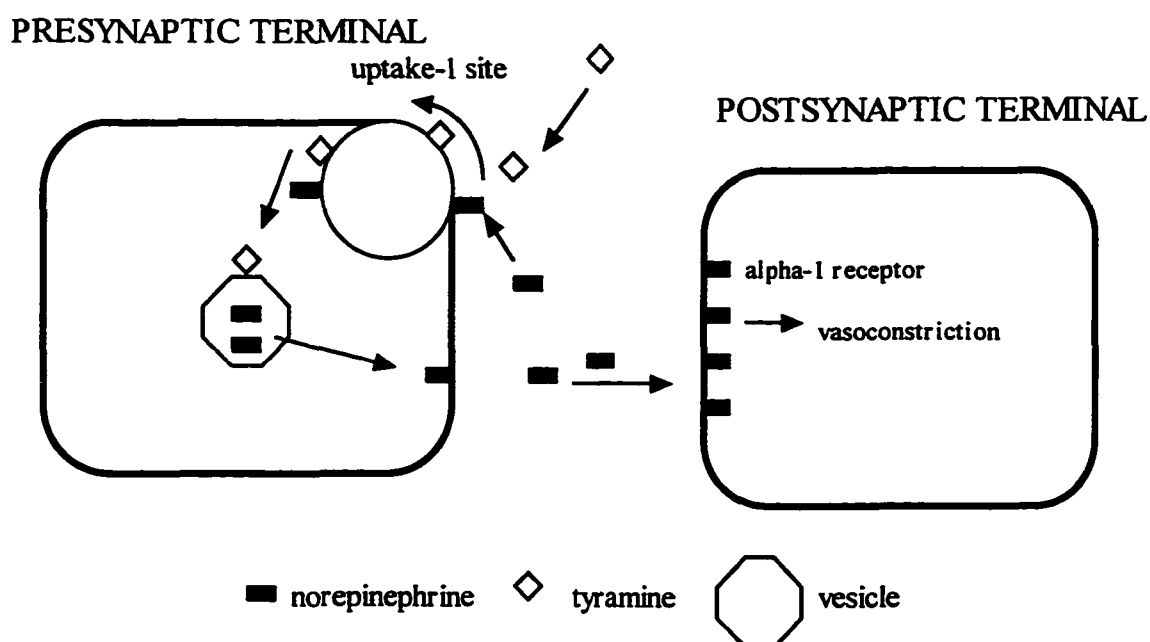


Figure 20. Diagrammatic representation of the pharmacological mechanism of tyramine. Tyramine displaces norepinephrine from presynaptic nerve terminals, norepinephrine binds to post-synaptic α_1 adrenoceptors to produce vasoconstriction.

Tyramine produced only a minimal response at a concentration of 10 μ M which may be due to limited norepinephrine containing presynaptic nerve terminals and lower α_1 adrenoceptor density than would normally be present in arterial smooth muscle. The weak response of the umbilical artery to norepinephrine supports this concept. At a 100 μ M tyramine concentration, there was a vasoconstrictive response, which was significantly greater in the presence of cocaine (10 μ M). The potentiation by cocaine produced a vasoconstrictive response to tyramine that was similar in magnitude to the KCl maximum. These results are similar to the findings of Gulati and Kelkar (1970); their study demonstrated a 20% potentiation of vasoconstrictive responses to tyramine in the isolated human umbilical cord perfusion model by cocaine. These results provide functional evidence that umbilical arteries are innervated and tyramine is producing its effects at an unidentified postsynaptic receptor. The paradoxical potentiation of tyramine-induced vasoconstriction by cocaine in umbilical arteries suggests that the effect of cocaine to inhibit its uptake into the nerve terminal is exacerbating this direct effect. It is also possible that the endothelium-dependent mechanism proposed by Zhang and Hu (1998) for serotonin potentiation by cocaine may be applicable to tyramine potentiation by cocaine as well.

Cocaine Enhancement of Vasoconstrictive Responses
to Norepinephrine and Serotonin

Exposure of the human umbilical artery to serotonin produced repeatable contractions that were significantly enhanced by cocaine. The concentration-effect curve for serotonin in the presence of cocaine was shifted to the left indicating greater sensitivity of the tissue to serotonin in the presence of cocaine (10 μ M). A vasoconstrictive response to norepinephrine was produced at very high concentrations and there was a significant potentiation of this response in the presence of cocaine. Cocaine also produced a leftward shift of the concentration-effect curve to norepinephrine indicating enhanced sensitivity of the tissue to norepinephrine in the presence of cocaine as well.

These results are analogous to the findings of Nair and Dyer (1974), who observed cocaine potentiation of serotonin and norepinephrine contractions in guinea pig umbilical artery strips. Gulati and Kelkar (1970) noticed that norepinephrine but not serotonin effects were potentiated by cocaine in the umbilical cord perfusion model. Dyer (1970) as well as Zhang and Hu (1998) produced cocaine potentiation of serotonin contractions in isolated ovine umbilical arteries. Dyer (1970) concluded that cocaine potentiation was produced by inhibition of neuronal reuptake of serotonin based on significant decrease of ^3H -serotonin uptake. Dyer (1970) also noted that the addition of desmethylinipramine

along with cocaine did not produce an exacerbation of this effect. Zhang and Hu (1998) attributed cocaine potentiation of serotonin contractions to the presence of endothelium. In their study, removal of the endothelium abolished the enhanced contractile effect of serotonin in the presence of cocaine. It is unclear whether cocaine is primarily inhibiting the reuptake of norepinephrine and serotonin into the presynaptic nerve terminal, limiting the major means of inactivation for these neurotransmitters and/or by preventing serotonin-stimulated release of endothelial-relaxing factor as proposed by Zhang and Hu (1998) in the ovine umbilical artery.

Kopin et al. (1984) estimated the mean synaptic concentration of norepinephrine to produce a 50 mm Hg increase in mean arterial pressure in pithed rats to be 7 nM. They demonstrated that treatment with desipramine produced a 3-fold increase in this concentration; this is within the concentration range of norepinephrine we used and observed a vasoconstrictive potentiation with cocaine. Like cocaine, desipramine blocks neuronal uptake and this effect at a synaptic junction in vivo supports the clinical relevance of the effect we observed in vitro with similar concentrations of norepinephrine.

Bodelsson and Stjernquist (1995) did not observe a potentiation of norepinephrine in the presence of a 1 μ M (0.3 μ g/mL) concentration of cocaine, a therapeutic to subtoxic plasma concentration in humans (therapeutic range of 0.05-0.31 μ g/mL, Winek 1994; Ellenhorn et al. 1997). Our cocaine concentration of 10

μM ($3.0 \mu\text{g/mL}$), is comparable to a toxic to potentially lethal plasma cocaine level in humans (toxic to lethal range of $0.9\text{--}21 \mu\text{g/mL}$, Winek 1994; Baselt and Cravey 1995). This suggests that the degree of significant constriction in the umbilical artery is dependent upon cocaine concentration and is produced by relatively higher plasma concentrations of cocaine which may occur with prolonged abuse or toxic dosage. This situation is possible and especially relevant in the umbilical artery since pregnancy decreases the metabolism of cocaine and increases the maternal half-life of cocaine and its metabolites (Neale and Parks 1973). Plessinger and Woods (1990) found that progesterone is involved in producing greater cardiovascular sensitivity to cocaine. In their study they found that cardiovascular toxicity in non-pregnant ewes was increased when progesterone was administered intramuscularly. Progesterone also increases the sensitivity of contractile alpha adrenoreceptors. Since progesterone concentrations increase with gestation, if the effects of cocaine are potentiated by progesterone, then cocaine use by the mother in the later stages of pregnancy would pose potentially greater risk for toxic vasoconstrictive effects.

Serotonin is a potent vasoconstrictor of umbilical arteries and cocaine has been shown to inhibit the neuronal reuptake of this substance in the brain (Ritz et al. 1990). We did produce a significant potentiation of the vasoconstrictive response to serotonin in the isolated human umbilical artery, demonstrating that

both norepinephrine and serotonin could be involved in the acute vascular toxicity of perinatal cocaine abuse. This information is particularly important considering that the vasoconstrictive effects of norepinephrine are augmented by serotonin (Van Neuten et al. 1985). The simultaneous potentiation of norepinephrine and serotonin by cocaine and augmentation of these effects by the neurotransmitters themselves could result in a toxic and potentially lethal pressor response of the umbilical artery to the fetus.

Potentiation of Norepinephrine and Serotonin Effects by Cocaine Metabolites

Cocaine metabolites shown to exhibit vasoconstrictive and hemodynamic effects similar to or exceeding those of cocaine were examined in this study for their ability to enhance the contractile responses to serotonin and norepinephrine in the isolated human umbilical artery. Benzoylecgonine, norcocaine, and cocaethylene were added to the preparation in the same manner described for cocaine, concentration-effect curves were constructed for serotonin and norepinephrine in the presence or absence of each metabolite. The effects of these metabolites were compared to cocaine by calculating the difference between area under the curve values for serotonin and norepinephrine with or without cocaine or metabolite; these differences were compared using a multiple comparisons analysis of variance and Fisher's LSD.

Benzoylecgonine (10 μ M) decreased in the maximum response to serotonin in this study by as much as 36%. The human umbilical artery was less sensitive to serotonin in the presence of benzoylecgonine as shown by the significant decrease in the area under the concentration-effect curve. Benzoylecgonine did not produce significant effects on norepinephrine responses in this study. There was a leftward shift of the EC15 in the concentration-effect curve for norepinephrine in the presence of benzoylecgonine, but the maximum response remained unchanged. Benzoylecgonine does produce effects alone *in vivo* and *in vitro*. These effects include increased cerebral vascular resistance in fetal lambs (Covert et al. 1994) and vasoconstrictive effects on fetal pig cerebral arterioles (Kurth et al. 1993). An *in vitro* study conducted by Madden and Powers (1990) using isolated cat cerebral arteries also demonstrated vasoconstrictive effects produced by benzoylecgonine. Norepinephrine induced constriction of the fetal pig arterioles was not potentiated by benzoylecgonine in the study by Kurth et al. (1994). Consistent with the serotonin findings in the present study, Covert et al. (1994) did notice an increase in fetal carotid blood flow in response to benzoylecgonine (peak serum concentration 3.61 mg/ml) administration in one ewe.

Norcocaine produced a significant potentiation of the maximum norepinephrine response and increased the sensitivity of the tissue to norepinephrine. Consistent with results produced in fetal pig cerebral arterioles,

norcocaine was able to significantly potentiate norepinephrine constriction of the vessels and produced a significant decrease in arteriolar diameter (Kurth et al. 1994). No significant effects were observed for the response to serotonin in the presence of norcocaine (10 μ M). There was an increase in the maximum response to serotonin, but this effect was not statistically significant due to variation in tissue responsiveness.

In this study cocaethylene did not produce significant increases in norepinephrine or serotonin vasoconstrictive responses of the human umbilical artery. Kurth et al. (1994) observed significant potentiation of norepinephrine induced constriction of fetal pig cerebral arterioles by cocaethylene and the authors reported cocaethylene to be a more potent constrictor of these arterioles than cocaine itself. Although the maximum response to norepinephrine in the presence of cocaethylene (10 μ M) was increased almost as considerably as that of norepinephrine with cocaine in this study, variation among tissue responses may have masked the statistical significance of cocaethylene enhancement.

Calculations of cumulative effects between serotonin concentration-effect curves before or after exposure of the tissues to cocaine or a metabolite revealed that effects of cocaine, norcocaine, and cocaethylene were similar. The effect of benzoylecgonine was significantly less than other metabolites due to decreased sensitivity of the tissue to serotonin in the presence of this metabolite. For

norepinephrine mediated contractions, calculated differences revealed that the effects of cocaine, norcocaine, benzoylecgonine and cocaethylene were not significantly different.

Effect of Methoxamine in the Human Umbilical Artery

The response to norepinephrine produced in this study was minimal, and methoxamine, a potent α_1 receptor agonist did not produce a vasoconstrictive response the human umbilical artery. This suggests that norepinephrine is producing its effect through receptors other than α_1 adrenoceptors. It is also unlikely that norepinephrine produces constriction of the umbilical artery through stimulation of α_2 adrenoceptors since Tuncer et al. (1985) were unable to produce contraction of human umbilical artery strips with clonidine, an α_2 selective agonist.

Ketanserin Attenuation of Cocaine Effects

We found that cocaine potentiates the maximum response to serotonin by as much as 28%. This is indicative of a significant role for serotonin in the perinatal toxicity which is seen with cocaine abuse. Tuncer et al. (1985) demonstrated antagonism of serotonin concentration-effect curves by ketanserin and mianserin in the human umbilical artery. Although the pA_2 values reported for these antagonists were lower than those reported in other tissues, the parallel rightward

shifts in the concentration-effect curves indicate that ketanserin may be useful in pathological serotonin-mediated contractions of the umbilical artery.

In these experiments ketanserin was able to antagonize the response to serotonin in the presence of cocaine, indicating that serotonin₂ receptors are involved in the vasoconstrictive response to serotonin potentiated by cocaine. Because serotonin is a very potent constrictor of umbilical arteries and cocaine potentiates this effect, ketanserin may be a useful agent in the treatment of acute perinatal cocaine toxicity which involves constriction of placental, uterine, and umbilical vasculatures.

We have also found that cocaine is able to produce a potentiation of norepinephrine-mediated vasoconstriction. Cocaine does potentiate the maximum response to norepinephrine by 64% (as noted in this study), however norepinephrine is not a very potent constrictor of umbilical arteries alone (Bodelsson and Stjernquist 1995). Methoxamine had no vasoconstrictive effect on isolated human umbilical arteries in our preparation. This indicates that norepinephrine may not be acting through α_1 receptors in this tissue. Based on the responses to phenylephrine and the antagonism of prazosin and rauwolscine at α -adrenoceptors, Bodelsson and Stjernquist (1995) concluded that norepinephrine was producing its effect through α_1 and α_2 adrenoceptors. In the same study, the authors noted that methysergide, a nonspecific serotonergic

antagonist, antagonized the concentration-effect curve of oxymetazoline, an α_2 agonist. In this study we examined the concept that norepinephrine may be exerting its effects through serotonin₂ receptors. We found that ketanserin antagonized the potentiation of norepinephrine by cocaine. The concentration-effect curve to norepinephrine with cocaine was shifted 60-fold to the right in the presence of ketanserin and cocaine, which was a complete attenuation of the potentiation produced by cocaine. This indicates that norepinephrine stimulation of serotonin₂ receptors may be a significant component of the enhanced response to norepinephrine produced by cocaine.

We have demonstrated that the vasoconstrictive effects of both norepinephrine and serotonin are potentiated by cocaine. The toxic potential of this situation is magnified by the concept that simultaneous stimulation of α -adrenoceptors and serotonin receptors is not merely additive but results in potentiation (Van Nueten et al. 1985). Since ketanserin is able to antagonize cocaine potentiation of the responses to both norepinephrine and serotonin in the umbilical artery, this suggests a clinical role for ketanserin in the treatment of acute perinatal cocaine toxicity situations.

³H-Ketanserin Membrane Binding Studies

In this study the binding characteristics of ³H-ketanserin were determined in the membrane fraction of homogenized human umbilical artery sections.

Umbilical artery membrane fractions were paired to the tissues used in the ketanserin attenuation contractile studies. Specific and saturable binding of ³H-ketanserin was produced, the K_D and B_{max} values calculated from the results of this study were much higher than those reported by Hu and Zhang (1997) for ³H-ketanserin in the ovine uterine artery (K_D $1.46 \pm .38$ nM and B_{max} 31.9 ± 5.9 fmol / mg protein). Similar results were obtained by Jazayeri et al. (1989) in the rat aortic smooth muscle cell (K_D 1.63 ± 1.3 , B_{max} 69 ± 22 fmol / mg protein). Indicating a very dense serotonin₂ receptor population in the human umbilical artery but lower affinity of ketanserin for the receptors.

The specific binding of the radioligand was also reversible and could be displaced by increasing concentrations of mianserin, serotonin and tyramine. Norepinephrine was not able to displace the radioligand even at 10 μ M concentrations. Consistent with this result, Jazayeri et al. (1989) were unable to displace ³H-serotonin with adrenergic agents in rat aortic smooth muscle cells. Paradoxical to the observations of the findings in the contractile studies, where tissues were sensitive to ketanserin attenuation of cocaine potentiated norepinephrine contraction, norepinephrine was unable to displace ³H-ketanserin

in the membrane binding studies. In the contractile studies, ketanserin with cocaine was unable to antagonize the concentration-effect curve for norepinephrine alone, consistent with the inability of norepinephrine to displace ^3H -ketanserin.

The concentration-dependent displacement of ^3H -ketanserin by tyramine is a novel finding. While tyramine is reported to produce direct vasoconstrictive effects in the umbilical artery (Gulati and Kelkar 1970) a receptor type for this effect has never been described. Tyramine concentration-displacement curves fit best to the two-site competition model indicating that tyramine binds at two serotonin₂ subtypes with very low affinity. In the contractile studies, tyramine produced vasoconstriction at a concentration of about 10 - 100 μM , and the K_D calculated for tyramine at the second site 36.2 μM is within this concentration. This provides good evidence that tyramine is producing its contractile effects through this receptor site, a serotonin₂ subtype.

Serotonin concentration-displacement curves also fit best to the two-site competition model indicating that serotonin binds at two serotonin₂ receptor subtypes. The K_D values for serotonin in this tissue (K_D 2.8 and 2700 nM) were higher than those for serotonin in cultured rat aortic smooth muscle (K_D 0.4 and 80 nM) indicating lower affinity of serotonin for serotonin₂ receptors in human umbilical arteries. Based on the contractile responses to serotonin in the present

study, the functional site for serotonin has a K_D of 2.8 nM in human umbilical arteries.

Since norepinephrine (10 μ M) did not displace 3 H-ketanserin, the vasoconstrictive effects of norepinephrine are probably not produced through serotonin₂ receptors. This conflicts with the results of the ketanserin contractile studies because cocaine potentiation of norepinephrine was attenuated by ketanserin. One explanation for this conflict might be that norepinephrine is producing its effects through another serotonin receptor subtype for which ketanserin has some affinity.

CHAPTER 5

SUMMARY AND CONCLUSIONS

1. Tyramine produced a vasoconstrictive effect that was potentiated by cocaine in the isolated human umbilical artery. Cocaine enhancement of tyramine effects indicates that neuronal uptake of tyramine is not necessary for its sympathomimetic effect.
2. Cocaine potentiation of both serotonin and norepinephrine vasoconstrictive responses indicates that the role of the umbilical artery in the vasoconstrictive toxicity of perinatal cocaine abuse is significant. The mechanism whereby cocaine produces its potentiating effects remains unclear.
3. Norcocaine potentiation of norepinephrine responses provides evidence that this cocaine metabolite may play a role in exacerbation of the vasoconstrictive complications attributed to perinatal cocaine abuse. Calculated effects of cocaine and its metabolites on norepinephrine induced vasoconstriction revealed no significant differences. This indicates that in

addition to cocaine and norcocaine, cocaethylene and benzoylecgonine may contribute to umbilical artery toxicity.

4. Cocaine metabolites norcocaine and cocaethylene did not produce significant enhancement of the serotonin contractile response. Maximum responses to serotonin were decreased in the presence of benzoylecgonine. It is unclear why benzoylecgonine decreased the sensitivity of this tissue to serotonin.
5. Methoxamine did not produce vasoconstriction of the human umbilical artery. This indicates that norepinephrine effects are unlikely to be mediated through α_1 receptors in this tissue.
6. Ketanserin abolished the potentiation of serotonin and norepinephrine responses by cocaine. Based on the findings of these contractile studies, cocaine potentiation of serotonin and norepinephrine responses is mediated through serotonin₂ receptors.

7. Binding characteristics of ^3H -ketanserin reveal a very high K_D for ketanserin in this tissue. Serotonin displaced ketanserin with very low affinity for serotonin₂ receptors. Since tyramine was able to displace ^3H -ketanserin in the membrane binding studies and its action is not dependent on neuronal uptake, tyramine may produce this response through postsynaptic serotonin₂ receptors. Norepinephrine did not displace ^3H -ketanserin from the membrane fraction in this assay.

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APPENDICES

APPENDIX A
ETSU and JCMC IRB Approved Protocol

Effects of Cocaine, Norcocaine, and Cocaethylene on the Isolated Human
Umbilical Artery and Vein and the Influence of Progesterone

Research to date indicates that infants delivered to cocaine or cocaine and ethanol abusing mothers have displayed increased incidence of neurological complications as well as other congenital anomalies. Cocaine use during pregnancy has been known to cause deleterious effects on placental function (decreased blood flow and nutrient transport) and on the fetus (growth retardation, SIDS, and teratologic effects) (Bingol et al. 1987), as well as on the pregnancy itself (spontaneous abortion, abruptio placentae, and premature delivery) (Acker et al. 1983). These complications have generally been attributed to the sympathomimetic and vasoconstrictive actions of cocaine and its metabolites (Covert et al. 1994). The primary pathophysiological action of cocaine and its other metabolites is related to the onset of hypertension and tachycardia. The etiology is directly related to increases in circulating norepinephrine (NE), due to a block of the transport of NE back into nerve terminals (Furchgott et al. 1963). This results in increased stimulation of alpha-1 and alpha-2 receptors by NE which causes vasoconstriction (Bayorh et al. 1983).

Cocaine is extensively metabolized in the plasma and liver, and its metabolites are cleared from the plasma more slowly than cocaine itself (Jatlow

1987); this includes cocaethylene (Jatlow et al. 1991), a toxic metabolite formed by the liver when cocaine and ethanol are used concomitantly (Boyer and Petersen, 1991). Cocaethylene is purported to be more toxic than cocaine and exhibits a lower LD50 in mice (Hearn et al. 1991). In one experiment involving porcine fetal cerebral arteries, cocaethylene was able to vasoconstrict these vessels more efficaciously than cocaine. In the same study, norcocaine, (although a minor metabolite of cocaine), was shown to constrict these vessels as efficaciously as cocaine itself (Kurth et al. 1993).

The toxicity of cocaine and its metabolites is greater due to decreased activity of cytochrome P₄₅₀ enzymes during pregnancy and under the influence of progesterone (Neale and Parks, 1973). Thus, progesterone increases enzymatic demethylations thereby increasing plasma concentrations of cocaine metabolites which have longer half-lives than cocaine and increases vascular reactivity to alpha-adrenergic receptor agonists. This suggests that pregnancy would increase cocaine sensitivity by increasing the formation of norcocaine and decreasing norcocaine metabolism. In one study, the cardiovascular toxicity to cocaine was shown to be increased in the presence of progesterone in nonpregnant ewes (Plessinger and Woods et al. 1990).

Numerous studies which have been conducted in animal models and in humans indicate that cocaine and its metabolites are rapidly transferred across the

placenta. Cocaine and its metabolites are transferred almost as rapidly as antipyrine, a substance which is used as a marker for placenta flow (Schenker et al. 1993). Although the placenta does contain considerable amounts of smooth endoplasmic reticulum and acetylcholinesterase as well as butyrylcholinesterase activity has been confirmed in human placenta (Koshakji et al. 1974), there has been no transplacental metabolism of cocaine observed to date.

The toxicology and teratology of cocaine during pregnancy and in the fetus can in part be attributed to vasoconstriction of the placenta (Mahalik et al. 1984). The vasoconstriction of the placental vasculature has been postulated to cause a decrease in uterine blood flow. Conversely, it has also been postulated that it is the vasoconstriction of the uterine vasculature initially which results in the decrease in blood flow to the placenta (Morgan et al. 1991). Conclusive data has yet to be presented to describe the true nature of this relationship. This decrease in blood flow has been proven to be the result of NE mediated vasoconstriction. This has been shown experimentally by incorporating tropacocaine, an agent which does not inhibit the reuptake of NE into the nerve terminal. In cocaine-exposed animals, there was a decrease in the amount of radio-labelled sodium transferred to the embryos. However, in the tropacocaine-exposed animals the transfer of the isotope was not reduced significantly (Mahalik et al. 1980). In response to intravenous cocaine administered to the uterine vasculature of the pregnant ewe

(Woods et al. 1987 and Moore et al. 1986), and to the pregnant nonhuman primate (Morgan et al. 1991), a dose-dependent reduction of uterine blood flow has been observed. This results in increased blood pressure and restricts the transfer of essential nutrients and oxygen across the placenta.

There have been few studies conducted which attempt to delineate the contribution of umbilical vasculature constriction to the fetal pathogenicity observed in human epidemiological data and in the animal model as a result of the administration of cocaine and its metabolites. Zhang and Dyer (1991) were able to produce dose-dependent contractions of the umbilical vein with NE, but did not observe this effect in the umbilical artery. They also observed that neither the EC_{50} nor the maximal contraction of the umbilical vein to NE were affected by cocaine, in contrast, tissue responsiveness in the vein to serotonin was potentiated 8.9 fold by cocaine. Serotonin (5-HT) seems to illicit stronger vasoconstrictive responsiveness than epinephrine (EPI) or NE (even at large concentrations) in umbilical arteries (Yoshikawa and Chiba, 1991). Thus it becomes very significant to investigate the effects of cocaine and its metabolites on this type of vasculature in the presence of 5-HT, NE, EPI, and dopamine (DA).

Unlike cocaine which acts at both dopaminergic and serotonergic reuptake sites (Ritz et al. 1990), cocaethylene acts preferentially to block reuptake at dopaminergic nerve terminals (Woodward et al. 1991). Therefore, the first

objective of this study will be to evaluate the involvement of cocaine, cocaethylene, and norcocaine in potentiating the vasoconstrictive responses of the isolated umbilical artery and vein to NE, 5-HT, EPI, and DA. Another point of interest arises when it is considered that ethanol, a vasodilator, must be circulating in order for the formation of the toxic metabolite cocaethylene. The effect of this compound on the vasoconstrictive effects of cocaine and its metabolites will also be evaluated in this model. Also, since it has been observed that the presence of progesterone increases the cardiovascular toxicity of cocaine, this effect will be evaluated in this model along with the toxic metabolites, norcocaine, and cocaethylene. If this toxicity is observed in this model, studies will then be conducted using Mifepristone (RU486), a progesterone antagonist, to determine if this effect can be blocked.

Materials and Methods

In order to determine the effect of cocaine, norcocaine (NC), and cocaethylene (CE) on the contractile activity of isolated human umbilical arteries and veins, umbilical artery and vein rings will be prepared for isometric tension recording and exposed to pharmacologically relevant concentrations of these agents by the method of Saade, et al.1995. Briefly, umbilical cords will be collected after vaginal or cesarean delivery and immediately placed in cold Krebs-bicarbonate solution. Arteries and veins will be dissected and cut into rings about

4 mm in length. The rings will be mounted in organ baths containing Krebs-bicarbonate solution at 37°C on stirrups which will be calibrated for isometric tension. A gas mixture of 95% oxygen and 5% carbon dioxide will be used to maintain tissue viability. Vascular ring tension will be equilibrated to about 2 grams or optimal tension depending on the responsiveness of the tissues. The rings will be contracted with KCl to confirm tissue viability and responsiveness. The contractile response of each ring to the specific neurotransmitter, either NE, 5-HT, EPI, or DA will be used as the reference to then be calculated as a percentage of its reference contraction. Each tissue will serve as its own control for comparison of cocaine, NC and CE effects. A minimum of four and a maximum of eight replicates will be done of each dose response curve pending response variation for statistical significance.

Experimental Protocol

In order to address all of the aforementioned aims, this project will be divided into four parts. The first experiment will be conducted to determine the concentration of Cocaine, NC or CE at which the dose response curves (10^{-7}M - 10^{-5}M) to the neurotransmitters NE, EPI, DA, and 5-HT produces the greatest shift in the EC_{50} to potentiate the response to these neurotransmitters. Tissues will be exposed to concentrations of metabolites beginning at 10^{-7}M for at least 45 minutes prior to administration of the neurotransmitters.

In order to answer the question of possible attenuation of the vasoconstrictive response of the tissue to Cocaine, NC and CE by ETOH, the second experiment will be performed to construct a dose response curve to ethanol (25, 50, 100, 200 and 300 mg/dL) in the presence or absence of each of these drugs. The attenuation of the contractile response produced by ETOH will be expressed as a percentage of the maximal contraction observed from each compound. These dose response curves will be constructed in the presence of those doses of either cocaine, NC or CE which produced the greatest shift of the EC_{50} in the dose response curve for each of the neurotransmitters.

The next set of experiments will be conducted to determine what effect exogenously applied progesterone will have on cocaine, NC or CE induced vasoconstriction. A dose response curve will be constructed for progesterone at concentrations which are characteristic of the luteal phase of the menstrual cycle (mean 43 nmol/L, range 6-79.5 nmol/L) and the three trimesters of pregnancy: 7-13 weeks (mean 86 nmol/L, range 32.6-139.9 nmol/L), 14-37 weeks (mean 162 nmol/L, range 62.0-262.4 nmol/L), and 30-42 weeks (mean 467 nmol/L, range 206.7-728.2 nmol/L). This dose response curve will then be reconstructed in the presence of cocaine, NC and CE and 50% dilatory dose of ETOH for comparison to the initial curve to determine if there is an interactive response of progesterone with these substances.

Mifepristone (RU486) will be used in the final set of experiments to determine if the enhanced vasoconstriction of cocaine, NC and CE mediated by progesterone can be blocked, if this effect is observed for progesterone. The isolated arteries and veins will be pretreated with single competitively antagonistic doses of Mifepristone, then exposed to the aforementioned doses of progesterone alone and with addition of 50% contractile doses of cocaine, NC and CE. The attenuation of Mifepristone will be calculated as a percentage of the maximum tension produced in the presence of progesterone for each compound.

APPENDIX B
Informed Consent

INFORMATION ABOUT: Effects of Cocaine, Norcocaine, and Cocaethylene on the Isolated Umbilical Artery and Vein, and the Influence of Progesterone.

INVESTIGATORS: Kenneth E. Ferslew, PhD., Tessa L. Long, Peter J. Rice, PhD., and Frederick R. Jelovsek, M.D.

CONSENT FORM

I understand that the purpose of this research project is to learn more about how the body works and changes in response in pregnancy, medicines, and disease. After delivery and detachment of the umbilical cord, we will examine the response of nerve chemicals in the blood vessels to drugs of abuse. Also, we will try to determine if hormones present during pregnancy will enhance the effects of these drugs on the blood vessels.

I understand that my involvement in the study will be my consent for the umbilical cord (after delivery) to be used in this project. The umbilical cord which would normally be discarded after birth will instead be taken to the laboratory for experimentation.

Approximately 50 umbilical cords will be used in this study at the Johnson City Medical Center Hospital and the E.T.S.U. Department of Pharmacology, Quillen College of Medicine.

All medical procedures are required as part of my treatment. I understand that there are no additional risks associated with this study.

I understand that there are no benefits to me as a participant in this study.

The purpose of the study is to learn more about how the body functions normally and under the influence of drugs. My participation may benefit others in the future. There is no additional cost for participating in this study.

I understand that my participation is voluntary. I realize that refusal to participate will in no way jeopardize benefits or treatments to which I am otherwise entitled. I am free to withdraw at any time without penalty or loss of benefits to which I am entitled. I understand that I may withdraw by notifying Tessa L. Long at 439-6208 or Kenneth E. Ferslew, PhD. at 439-6274. If I have any questions about the study I may contact either of these people as well.

Any questions about my rights as a research subject can be addressed to the ETSU Institutional Review Board (423/439-6134). East Tennessee State University does not provide compensation for medical treatment other than emergency first aid for any injury which may occur as a result of your participation as a subject in this experiment, claims arising against ETSU or any of its agents or employees may be submitted to the Tennessee Claims Commission for disposition to the extent allowable as provided under TC Section 9-8-307. Additional information concerning this may be obtained from the Chairman of the Institutional Review Board (IRB), 439-6134.

INFORMATION ABOUT: Effects of Cocaine, Norcocaine, and Cocaethylene on the Isolated Umbilical Artery and Vein, and the Influence of Progesterone.

INVESTIGATORS: Kenneth E. Ferslew, PhD., Tessa L. Long, Peter J. Rice, PhD., and Frederick R. Jelovsek, M.D.

I understand that a copy of the results of this experiment will be deposited on file in the Department of Toxicology, West Side of Memorial Center W269 on the ETSU campus for a period of at least 10 years after completion of the research project. I understand that results of this study may be published and/or presented at meetings. I understand that study records are accessible to: ETSU IRB, Department of Health and Human Services, and coinvestigators. I also understand that my medical records will be maintained in strictest confidence according to current legal requirements, and will not be revealed unless required by law, except as noted above.

By signing below, I certify that I have read or had this document read to me and I have been given a copy. I have been given the opportunity to ask questions and to discuss my participation with the investigator. I freely and voluntarily choose to participate in this research experiment.

Patient's Signature

Date

Witness' Signature

Date

Investigator's Signature

Date

APPENDIX C

Modified Krebs-Henseleit Buffer

Modified Krebs-Henseleit Buffer

Reagents

Stock A (10X Concentrate)

	<u>Molecular Wt.</u>	<u>Mass (g)</u>
Sodium Chloride	58.44	132.00
Potassium Chloride	74.56	7.083
Calcium Chloride • 2H ₂ O	147.07	7.412
Magnesium Sulfate • 7H ₂ O	246.48	5.866
Dextrose	180.16	20.00

Dissolve in Deionized Water 2000ml

Stock B (10X Concentrate)

Potassium Hyd. Phosphate	136.09	3.212
Sodium Bicarbonate	84.01	42.00

Dissolve in Deionized Water 2000ml

Procedure

1. For 2 liters of buffer, add 200 ml of stock A and B to a volumetric flask and bring up to volume with deionized water.
2. Addition of deionized water (about 500 ml) between the mixing of stock aliquots prevents precipitation of calcium salts.
3. The buffer is aerated with 95%O₂/ 5% CO₂ for about 30 min before use.

APPENDIX D

Modified Krebs-Henseleit Buffer 65 mM Potassium Replacing Sodium

Modified Krebs-Henseleit Buffer 65mM Potassium Replacing Sodium

Reagents

Stock A (10X Concentrate)

	<u>Molecular Wt.</u>	<u>Mass (g)</u>
Sodium Chloride	58.44	15.741
Potassium Chloride	74.56	23.792
Calcium Chloride●2H ₂ O	147.07	1.853
Magnesium Sulfate●2H ₂ O	246.48	1.466
Dextrose	180.16	5.00

Dissolve in Deionized water	500ml
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Stock B (10X Concentrate)

Potassium Hyd. Phosphate	136.09	3.212
Sodium Bicarbonate	84.01	42.00

Dissolve in Deionized water	2000ml
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Procedure

1. For 1 liter 65 mM K⁺ buffer, add 100 ml of stock A and B and bring up to volume with deionized water.
2. Addition of deionized water (about 500 ml) between addition of stock aliquots prevents the precipitation of calcium salts.
3. The buffer is aerated with 95%O₂/ 5% CO₂ for about 30 min before use.

APPENDIX E

Bradford Protein Assay

Bradford Protein Assay

Principle

The Bradford method is based on the shift in absorption maximum (from 465 to 595 nm) which occurs when protein binds to the dye Coomassie Brilliant Blue G-250. This protein assay is very reproducible and rapid; there is little interference from cations such as sodium or potassium nor from carbohydrates. Interfering color can be produced by large amounts of detergents.

Materials

Microtiter plates

Equipment

Spectra Max 340 Microplate Spectrophotometer
(Molecular Devices, Sunnyvale, CA)

Reagent

Bio-Rad Protein Assay Dye Reagent Concentrate
Bovine Serum Albumin (BSA) (1mg/ml)

Procedure

1. Prepare 100 μ l BSA standards containing 0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 μ g. Use 100 μ l sample of unknown concentration (0.25-10 μ g) protein.
2. Add 150 μ l of diluted (1 part Reagent plus 2 parts water) Bio-Rad Dye and mix well. The reaction is complete within 2 min and is stable for 1 hour.
3. Read absorbance at 595 nm against a control with zero protein.

Calculations

A standard curve is constructed using bovine serum albumin and used to convert absorbance at 595 nm to an equivalent protein concentration.

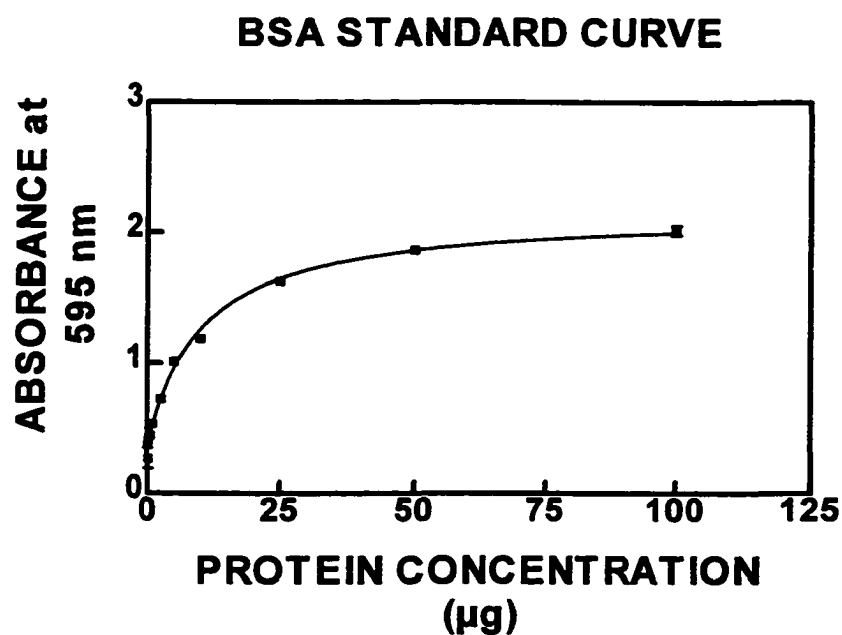


Figure 21. Standard curve for Bradford protein assay. Data are represented by the mean of 4 replicates.

VITA

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Teaching
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 Autonomic Pharmacology: Computer Simulated Dog Lab
 Antihyperlipidemic Drugs: Lecture
 Individual Tutoring, entire course

Honors: Most Outstanding Poster Presentation Award at the Southeastern Society of Toxicology 1997 13th annual meeting

Travel Award, Society of Toxicology 1998 37th Annual Meeting in Seattle, Washington

2nd Place Poster Presentation in the 14th annual East Tennessee State University Research Forum, Graduate Student division II.

Educational Research Award 1998 Society of Forensic Toxicology

Published Abstracts:

Long, T.L., Ferslew, K.E., Rice, P.J., Nwosu, U.C., and Jelovsek, F.R. Potentiation of the vasoconstrictive effect of serotonin, norepinephrine, and tyramine by cocaine on the isolated umbilical artery. *Tox Talk*, 1997, 21(4):9.

Long, T.L., Rice, P.J., Jelovsek, F.R., Jaekle, R.K., Nwosu, U.C., and Ferslew, K.E. Comparison of cocaine metabolites on vasoconstriction of umbilical artery rings to norepinephrine and serotonin. *Am. J Obstet. Gynecol.* 1998, 78(1)2:S34.

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Manuscripts Submitted for Publication:

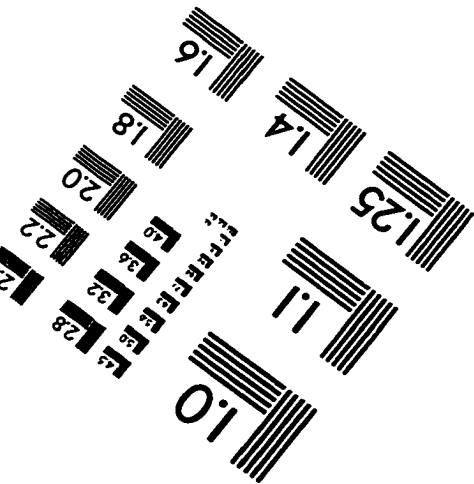
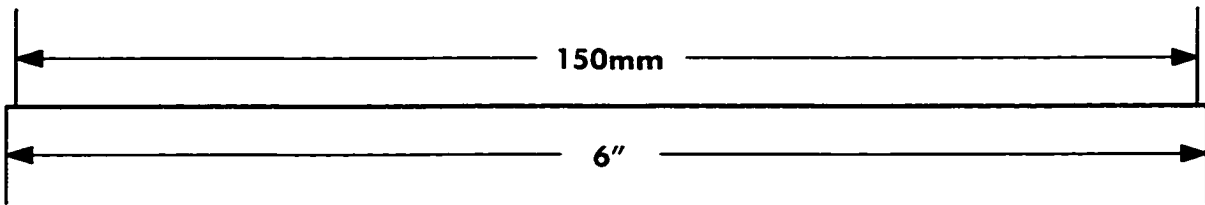
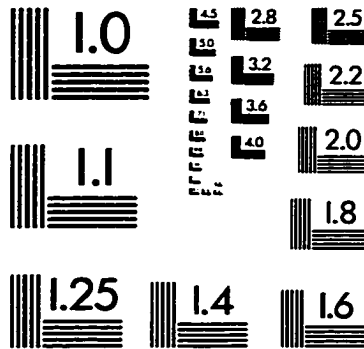
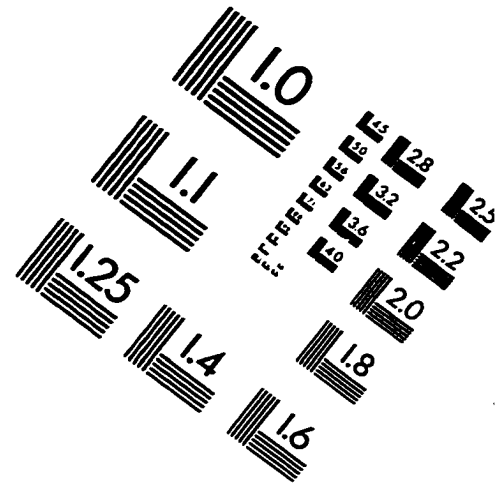
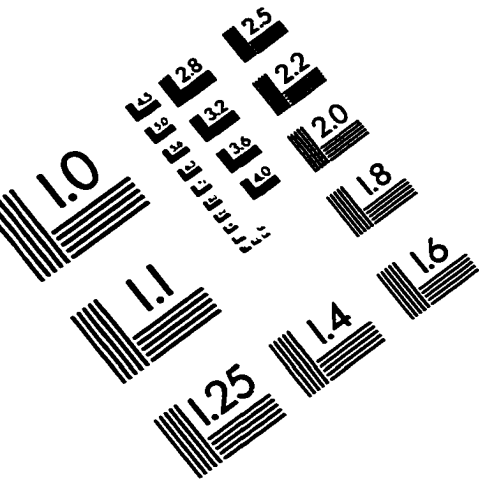
Cocaine potentiates the vasoconstrictive effect of serotonin, norepinephrine, and tyramine on the human umbilical artery. *Am. J Obstet. Gynecol.*

5HT₂ antagonist ketanserin blocks the vasoconstrictive toxicity produced by cocaine in isolated human umbilical arteries. *Tox Appl. Pharm.*

Displacement of ³H-ketanserin by serotonin, tyramine and mianserin from the human umbilical artery membrane fraction. *Pharm. Sci.*

Comparison of cocaine and metabolite effects on the vasoconstrictive response to norepinephrine and serotonin in the human umbilical artery. *J Pharmacol. Exp. Ther.*

IMAGE EVALUATION TEST TARGET (QA-3)



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